ANÁLISIS DE LOS FACTORES RESPONSABLES DE LA EVOLUCIÓN DE ANGIOSPERMAS DURANTE EL CUATERNARIO: UN ESTUDIO MACRO- Y MICROEVOLUTIVO EN *LINARIA* SECT. *SUPINAE*

José Luis Blanco Pastor

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Dr. Pablo Vargas Gómez, Investigador del Real Jardín Botánico de Madrid (CSIC) y **Dr. Concepción Ornosa Gallego**, Profesora Titular de la Universidad Complutense de Madrid

CERTIFICAN

Que los trabajos de investigación desarrollados en la Memoria de Tesis Doctoral: "Análisis de los factores responsables de la evolución de angiospermas durante el Cuaternario: un estudio macro- y microevolutivo en *Linaria* sect. *Supinae*", **son aptos para ser presentados por el Ldo. José Luis Blanco Pastor ante el Tribunal que en su día se designe, para aspirar al Grado de Doctor con mención Internacional por la Universidad Pablo de Olavide.**

Y para que así conste, y en cumplimiento de las disposiciones legales vigentes, extendemos el presente certificado a 20 de diciembre de 2013

Dr. Pablo Vargas Gómez

Dr. Concepción Ornosa Gallego

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A mis padres, mi hermana e Isa

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RESUMEN

En la presente memoria de tesis, se descubren patrones evolutivos de un grupo de plantas con flor formado por la sect. *Supinae* del género *Linaria* (Plantaginaceae). Se presentan cinco manuscritos científicos en los que se han utilizado distintos niveles taxonómicos (género, sección, subsección, especie y población) como hipótesis evolutivas. Cada manuscrito tiene objetivos específicos relevantes a distintas escalas espaciales y temporales. Se presta especial atención a las consecuencias evolutivas derivadas de las distintas estrategias reproductivas de las especies. Concretamente tras evaluar la monofilia de la sect. *Supinae*, se discuten los patrones evolutivos relacionados con la hibridación entre especies, la autogamia y la alogamia; además se analiza el papel de los polinizadores en la evolución de algunas especies alógamas. La introducción comienza describiendo herramientas fundamentales utilizadas en la presente memoria de tesis. Posteriormente se resumen el rol que pueden tener las diversas estrategias reproductivas en la evolución de las plantas con flor. Tras la presentación de cinco manuscritos, los resultados principales de esta investigación se discuten en el contexto de la evolución de plantas con flor durante el Cuaternario

SUMMARY

In this PhD report, evolutionary patterns in a group of flowering plants formed by sect. *Supinae* species of genus *Linaria* (Plantaginaceae) are disclosed. Five scientific manuscripts are presented in which distinct taxonomic levels (genus, section, subsection and species) are used as evolutionary hypotheses. Each manuscript has specific objectives at distinct spatial and temporal scales. The study focuses on evolutionary consequences derived from the diversity of reproductive strategies of the species. Specifically, after evaluating the monophyly of sect. *Supinae*, the evolutionary patterns related to hybridisation among species, autogamy and allogamy are discussed; also, the role of pollinators in the evolution of some allogamous species is analysed. The introduction starts describing basic tools used in this PhD report. Afterwards, it is summarized the role of the diverse reproductive strategies in the evolution of flowering plants. After the presentation of the five manuscripts, the main results of this research are discussed in the context of the evolution of flowering plants during the Quaternary.

INTRODUCCIÓN GENERAL

HERRAMIENTAS PARA EL ESTUDIO DE LA EVOLUCIÓN: TAXONOMÍA, SISTEMÁTICA, BIOGEOGRAFÍA, FILOGEOGRAFÍA Y MODELOS DE NICHO ECOLÓGICO

La **taxonomía** se define como la forma de organizar los individuos en base a información biológica. A principios del siglo XVIII los naturalistas europeos comenzaron el catálogo de la diversidad biológica siguiendo un esquema de clasificación desarrollado por el botánico sueco Carl Nilsson Linæus (Carlos Linneo, 1707-1778), que es el mismo que se sigue usando en nuestros días. Linneo introdujo la nomenclatura binomial y propuso un sistema de agrupamiento de especies de forma jerárquica, con grupos anidados en otros mayores. La taxonomía hoy en día sigue siendo una disciplina eminentemente empírica y descriptiva que acumula datos para generar hipótesis filogenéticas de las relaciones entre individuos y taxones.

La **sistemática**, ciencia que clasifica los organismos teniendo en cuenta su historia evolutiva, se ha desarrollado enormemente en los últimos 60 años. En la década de los 50 el entomólogo alemán Willi Hennig (1950) revolucionó la forma de considerar la clasificación de los seres vivos argumentando que debían clasificarse sobre la base de análisis filogenéticos que él mismo propuso. El uso inicial de caracteres morfológicos y una implementación manual para construir las filogenias mediante máxima parsimonia dio paso en la década de los 90 al uso de datos moleculares obtenidos a través de la reacción en cadena de la polimerasa (PCR) y métodos computacionales basados en máxima verosimilitud (Felsenstein 1981) e inferencia Bayesiana mediante cadenas de Markov (MCMC) (Yang & Rannala 1997). Este desarrollo, que llega hasta nuestros días, ha permitido un avance exponencial en la resolución del árbol de la vida. Más recientemente, las técnicas de datación por medio de reloj molecular relajado (e.g. Sanderson 1997; Drummond *et al.* 2006), que permiten una tasa evolutiva cambiante entre los linajes, y la calibración con fósiles han permitido establecer el marco temporal de las relaciones filogenéticas, lo cual ha sido fundamental para comprender como han evolucionado los linajes a través del tiempo.

Sin embargo, han aparecido nuevos desafíos para las reconstrucciones filogenéticas. Entre ellos, la gran dificultad en la revelación de las relaciones entre los linajes más terminales del árbol de la vida. Esta dificultad radica principalmente en la identificación de regiones moleculares variables, así como en el desajuste entre el árbol génico y el árbol de especies por reparto incompleto de los linajes de genes (*incomplete lineage sorting*) y/o eventos de hibridación/introgresión. La forma de abordar estos problemas es mediante la inclusión de múltiples genes y reconciliando el desajuste con distintos modelos y tests que tengan en cuenta estos procesos. Sin embargo, hasta el reciente desarrollo de la secuenciación de nueva generación, la obtención de múltiples genes ha sido una tarea muy compleja y aún existen numerosas dificultades analíticas cuando las especies estudiadas

han podido sufrir varios de estos procesos de forma conjunta (véase Kubatko 2009; Heled *et al.* 2013; Yu *et al.* 2013).

La **biogeografía** pretende explicar la distribución geográfica de los seres vivos teniendo en cuenta su historia evolutiva. Tradicionalmente la biogeografía ha propuesto dos procesos para explicar el origen de las distintas distribuciones de organismos o linajes emparentados: dispersión y vicarianza. En la última década del siglo XX se desarrollaron nuevos métodos de análisis biogeográficos basados en filogenias moleculares, lo que ha permitido examinar la influencia relativa de ambos procesos en la distribución de los organismos. Fredrik Ronquist (1997), incorporó además los procesos de duplicación y extinción a los modelos de dispersión y vicarianza. En estos modelos cada proceso recibe un coste según su probabilidad. Aplicando un análisis de máxima parsimonia como criterio de optimización, se puede discriminar entre distintos patrones biogeográficos. El uso de filogenias moleculares y su datación mediante el registro fósil y el uso de reloj molecular han permitido en los últimos años testar dispersion vs. vicarianza, siendo la primera más probable cuando la barrera es anterior a la divergencia y la última más probable cuando la barrera es de la misma edad que la divergencia (resumen de los distintos métodos biogeográficos en Ronquist & Sanmartín 2011). Esto ha demostrado la importancia de la dispersión a larga distancia en las especies demasiado jóvenes para que se vean afectados por episodios de vicarianza.

La **filogeografía** es una disciplina a caballo entre la biogeografía y la genética de poblaciones. Fue introducida por John Avise (1987, 2001) y continúa desarrollándose analíticamente hasta nuestros días (Templeton 1998; Posada *et al.* 2000; Beaumont & Panchal 2008; Lemey *et al.* 2009). Pretende analizar patrones a nivel intraespecífico (o en especies estrechamente emparentadas) utilizando secuencias de DNA y se caracteriza por permitir la reconstrucción de procesos biogeográficos recientes, como las fluctuaciones en el área de distribución durante las glaciaciones del Cuaternario. La filogeografía ha hecho uso reciente de la teoría de la coalescencia (Kingman 1982) para poder reconstruir la historia de las poblaciones, ya que son necesarios análisis específicos para averiguar si la similitud genética se debe a eventos de migración o a retención de polimorfismos ancestrales (Beerli & Felsenstein 2001; Hey & Nielsen 2007; Hey 2010). La historia demográfica de las poblaciones (fluctuaciones en el tamaño efectivo poblacional) también dejará una huella en el genoma de sus representantes actuales. Un amplio abanico de métodos permiten estimar contracciones y expansiones demográficas, pero solo recientemente se han conseguido inferir patrones cambiantes complejos mediante el uso de *skyline-plots* bayesianos (Pybus *et al.* 2000; Ho & Shapiro 2011). Estos métodos permiten cuantificar la relación entre la genealogía y el tamaño efectivo poblacional (el número de individuos que contribuyen a la descendencia en la siguiente

generación). De esta forma se pueden estimar procesos demográficos tales como cuellos de botella o expansiones demográficas rápidas a lo largo de la historia de la especie.

Las variables climáticas afectan a los patrones de distribución y a la demografía de las especies. En los últimos años han sido de especial relevancia los avances en el desarrollo de métodos de **modelización del nicho ecológico** (*Ecological Niche Modelling*, ENM). Estos métodos utilizan variables climáticas y datos de distribución actual de los organismos para modelar la tolerancia ecológica y hacer proyecciones de la distribución a climas pasados o futuros. La integración de las inferencias filogeográficas con el enfoque ecológico mediante análisis ENM ha resultado ser muy valiosa para contrastar hipótesis evolutivas (Carstens & Richards 2007; Richards *et al.* 2007; He *et al.* 2013).

ESPECIACIÓN SEGÚN EL ORIGEN GEOGRÁFICO DE LAS BARRERAS REPRODUCTIVAS: ESPECIACIÓN ALOPÁTRIDA, PARAPÁTRIDA Y SIMPÁTRIDA

La **especiación alopátrida** es la evolución de barreras reproductivas entre poblaciones que están separadas geográficamente, lo que impide el intercambio genético entre ellas. Estas barrearas pueden ser tanto físicas (topografía, masas de agua) como ecológicas (tipo de suelo, interacciones con otras especies). Dentro de la especiación alopátrida podemos encontrar casos de **vicarianza**, esto es, divergencia entre dos grandes poblaciones o **especiación peripátrida**, que sería la divergencia de una pequeña población (normalmente por efecto fundador) a partir de otra ampliamente distribuida. En la especiación alopátrida la migración entre poblaciones sería insignificante y por lo tanto no contrarrestaría la divergencia por deriva genética o por efecto de selección. La mayoría de biólogos evolucionistas consideran que la especiación alopátrida es la forma prevalente de especiación (Mayr 1963; Grant 1981; Coyne & Orr 2004). Las poblaciones alopátridas pueden expandir su área de distribución y entrar en contacto secundario (como por ejemplo en expansiones durante los ciclos glaciales). Ya que las expansiones de área de distribución han debido ser bastante recurrentes en el pasado, especies simpátridas actuales deben de haber especiado de forma alopátrida. Tras el contacto, las nuevas especies pueden volverse simpátridas pero sin producirse intercambio genético. Sin embargo, si las barreras reproductivas no son completas se podrá producir hibridación o introgresión.

La **especiación parapátrida** se produce cuando poblaciones espacialmente diferenciadas en un espacio continuo, pero con cierto grado de flujo genético, divergen y desarrollan aislamiento reproductivo. En la especiación parapátrida la presión selectiva debe ser mayor que en la especiación alopátrida para generar barreras reproductivas. Si estas presiones selectivas son suficientemente fuertes la especiación ocurrirá independientemente del grado de flujo genético. El flujo genético, sin embargo, podrá determinar la localización de la zona de diferenciación (Endler 1973).

La **especiación simpátrida** es la evolución de barreras reproductivas dentro de una población presuntamente panmíctica. A diferencia de la especiación alopátrida, la reducción en el intercambio genético se logra a través de un cambio evolutivo en las características de los organismos. Para que existan barreras reproductivas en simpatría deben generarse incompatibilidades precigóticas (e.g. morfológicas, fenológicas) o postcigóticas (e.g. alélicas o cromosómicas). En plantas los mecanismos comunes de especiación simpátrida serían: (i) la especiación por poliploidía y (ii) la especiación por recombinación (especiación híbrida homoploide). La autofecundación, propagación vegetativa, un mayor *fitness* o separación de nicho para las nuevas formas dará lugar a que éstas aumenten su

tamaño poblacional y den lugar a poblaciones viables (Fowler & Levin 1984; Rodríguez 1996; Rieseberg 1997). Otros modelos de especiación simpátrida se basan en selección disruptiva sexual o selección disruptiva sobre ciertos recursos pero sin cambios en el nivel de ploidía o sin producirse hibridación. La selección disruptiva sexual deberá ir acompañada por evolución de la divergencia ecológica para evitar la competición entre especies incipientes. También la selección disruptiva sobre recursos deberá ir acompañada por la evolución de la selección sexual, debido a que los alelos que actúen sobre el aislamiento sexual y los alelos que actúen sobre la disrupción del carácter no tendrían por qué ser los mismos. De esta forma se podría producir intercambio genético y recombinación entre especies incipientes (Coyne & Orr 2004).

PATRONES EVOLUTIVOS SEGÚN LOS SISTEMAS REPRODUCTIVOS

La evolución de los sistemas reproductivos ha atraído durante mucho tiempo a biólogos evolucionistas ya que determina la transmisión de genes a la siguiente generación y, por lo tanto, tiene un efecto crucial en la variabilidad genética y supervivencia de las poblaciones. En plantas, la transmisión de polen y semillas es el medio por el cual la información genética migra entre poblaciones. Conocer lo procesos de polinización y dispersión de semillas es fundamental para comprender los patrones evolutivos.

Hoy sabemos que los sistemas genéticos de las angiospermas han promovido la evolución hacia la reproducción alógama, con la mayor selección dirigida hacia las características florales (Wilson & Thomson 1996; Ayala *et al.* 2000). La explicación clásica de la evolución de caracteres florales en plantas polinizadas por animales ha sido la especialización hacia ciertos polinizadores para favorecer su atracción o la eficiencia en la transmisión de polen (Darwin 1862; Stebbins 1970; Faegri & Van der Pijl 1979). Sin embargo, mientras que algunas plantas con flor son completamente alógamas, otras dependen de la autogamia para asegurar la producción de semillas. Los estudios de los sistemas reproductivos en plantas han estado presentes de forma muy amplia en la literatura. Se han analizado los efectos relativos de la autogamia y alogamia en factores como la diversidad y estructuración genética, la colonización y la supervivencia de las poblaciones (Charlesworth & Charlesworth 1995; Richards 1997; Holsinger 2000). Además, el desarrollo en los últimos años de complejos modelos filogenéticos está permitiendo aclarar la influencia de los sistemas reproductivos en los patrones macroevolutivos (especiación vs. extinción, Goldberg *et al.* 2010; Goldberg & Igić 2012). Sin embargo aún encontramos ciertas dificultades como la correcta estimación de tasas de extinción a partir de filogenias moleculares o la inclusión de sistemas reproductivos mixtos en los modelos.

En los últimos años ha habido un gran interés en las causas y consecuencias evolutivas de las transiciones de alogamia a autogamia en gran medida por la frecuente aparición de este cambio en las angiospermas (Barrett 2002). Las especies autógamas son normalmente más homocigotas, tienen menos loci polimórficos y menos alelos por locus polimórfico y tienen un menor número de genotipos que las especies próximas alógamas (Ayala *et al.* 2000). Por lo tanto la autogamia debe reducir la capacidad de las poblaciones para responder a cambios ambientales a través de la selección natural. Sin embargo, se conoce que en circunstancias como la carencia de polinizadores específicos o ineficiencia en la transmisión de polen, la autofecundación y la consiguiente producción asegurada de semillas pueden ser beneficiosas (Baker 1966, 1967; Ashman *et al.* 2004). Las plantas autógamas están más adaptadas a circunstancias inmediatas pero son menos capaces de

adaptarse a cambios a largo plazo, si las comparamos con las especies alógamas. Por ello, los distintos modos reproductivos (alogamia vs. autogamia) históricamente se han asociado a distintas formas de vida (perenne vs. anual) y/o distintos hábitats (estables vs. inestables) (Stebbins 1957).

PATRONES EVOLUTIVOS EN ESPECIES AUTÓGAMAS

La autogamia ha evolucionado en muchos grupos diferentes de angiospermas a partir de ancestros alógamos. Esta transición debe haber sido favorecida bajo varias condiciones:

- En dispersiones a larga distancia a partir de un solo propágulo (*Baker's rule* (Baker 1955))
- Cuando la transmisión de polen por parte de los insectos polinizadores es ineficiente.
- Cuando hay limitación de polen, para reducir el gasto de gametos masculinos.
- Cuando los descendientes formados con polen externo son menos exitosos, ya que la autofecundación permite el control de la identidad parental en la descendencia.

Estos cuatro puntos se pueden agrupar en uno: la seguridad reproductiva (*reproductive assurance hypothesis*, Jain 1976).

- También, en poblaciones alógamas con individuos autocompatibles se puede producir selección automática o un alto coste de alogamia (Fisher 1941; Wyatt 1983; Holsinger 2000). En este caso, los genes que determinan la autocompatibilidad pueden seleccionarse y hacerse más abundantes ya que estos individuos pueden aportar su polen para formar genotipos tanto en sus mismos óvulos como en los óvulos de otros individuos.

La desventaja de la autofecundación habitual en una población es la disminución de heterocigosidad (y aumento de homocigosidad) que puede dar lugar a los siguientes procesos negativos:

- Con altos niveles de homocigosidad, los alelos recesivos letales u otros genes desventajosos pueden expresarse, dando lugar a depresión por endogamia.
- Se pierden los efectos de sobredominancia que ocurren como resultado de genes en estado heterocigoto que muestran un efecto de vigorosidad.
- La homocigosidad puede reducir la variabilidad que se genera por recombinación en líneas autógamas.

En la mayoría de los casos la selección de la autocompatibilidad se produce por la prevalencia de la seguridad reproductiva sobre la variabilidad genética en la población. A pesar de la pérdida de heterocigosidad, las líneas genéticas homocigotas podrán evolucionar por recombinación. Además, una reducida proporción de alogamia en poblaciones autógamas podrá dar lugar a un incremento sustancial de la variabilidad, y por lo tanto, de la capacidad adaptativa (Richards 1997). Una vez que la autocompatibilidad se establece como un modo exitoso de reproducción, las líneas autógamas pueden favorecerse secundariamente de mecanismos de eficiencia como la cleistogamia (polinización anterior a la antesis), o mecanismos de ahorro energético como la reducción en el tamaño de las flores, el número de granos de polen o el volumen de néctar.

PATRONES EVOLUTIVOS EN ESPECIES ALÓGAMAS

Evolución mediada por hibridación

La idea de que se pueden formar nuevas especies de plantas a través del cruzamiento entre especies (hibridación) fue ya propuesta por *Linneo* (Linnaeus 1760; véase también Roberts 1929), que indicó que la diversidad de especies dentro de un género se debería a la creación por *generación híbrida*. Posteriormente hubo una profusión de evidencias sobre hibridación en la literatura (e.g. Herbert 1847; Darwin 1859; Naudin 1862) hasta que Mendel (1866) declaró que la hibridación sería una característica de gran importancia para la historia evolutiva de las plantas ya que *híbridos constantes ganarían el estatus de nuevas especies*. La siguiente gran contribución a la idea de la especiación híbrida la hizo Winge (1917), quien señaló que las especies híbridas pueden formarse instantáneamente por la duplicación del complemento cromosómico (alopoliploidía). Esta hipótesis fue confirmada experimentalmente en otras especies y, por lo tanto, se ha reconocido la alopoliploidía como un modo importante de especiación en plantas con flor (Stebbins 1950). En cambio, la idea de especiación híbrida en ausencia de cambio de nivel de ploidía no se estableció hasta el trabajo de Müntzing (1930). Este autor indicó que una nueva población híbrida podía ser fértil y estable manteniendo el mismo nivel de ploidía que sus progenitores (especiación híbrida homoploide). El aislamiento reproductivo frente a ambos se podría producir mediante la inviabilidad de ciertas combinaciones cromosómicas (por inserciones y/o delecciones). Además de los factores cromosómicos, otros modelos han incorporado factores génicos o los precigóticos como generadores de aislamiento. Entre estos últimos se incluyen las preferencias de sustrato o polinizadores. La frecuencia de híbridos naturales tiene un rango entre el 6% y el 22% tras el análisis biosistemático en cinco grandes floras modernas (Ellstrand *et al.* 1996), unos valores que deben

representar una subestimación considerable teniendo en cuenta que no se habrán podido detectar las especies híbridas crípticas o las especies originadas por hibridación antigua (en las que no se tenga conocimiento de los progenitores). El análisis de marcadores moleculares es la herramienta más sólida para la identificación de taxones con origen híbrido, pero incluso mediante esta aproximación se pueden obtener resultados ambiguos. Un taxon puede compartir marcadores moleculares de taxones relacionados debido a la retención de alelos ancestrales tras la especiación, dando lugar al reparto incompleto de linajes génicos (*incomplete lineage sorting*), lo cual puede confundirse con la señal de hibridación. Incluso cuando la evidencia a favor de la hibridación es clara (debido al uso de múltiples marcadores moleculares), esta no podrá ser asociada a especiación híbrida debido a que la distinción entre especiación híbrida e introgresión genética es una tarea compleja (Rieseberg 1997).

La especiación híbrida homoploide se ha considerado un proceso poco probable ya que debe ocurrir en simpatría y sin favorecerse del aislamiento reproductivo vía poliploidia (Rieseberg 1997; Mallet 2007). De todos modos, muchas poblaciones híbridas homoploides probablemente se originaron tras un contacto secundario en un nuevo hábitat disponible (después de la expansión desde un refugio o de dispersión a larga distancia). Es más probable que dicha población evolucione hacia una nueva especie híbrida cuando un nuevo nicho ecológico está disponible y no es utilizado por las especies progenitoras. De hecho la hibridación y la introgresión pueden fomentar la variación genética permitiendo la adaptación a nichos extremos disponibles (Rieseberg *et al.* 2003). Por ello, se considera que la hibridación debe ser un catalizador no solo de la especiación sino también de la innovación evolutiva. Hoy en día se sabe que la hibridación e introgresión han sido recurrentes a lo largo de la historia evolutiva de las angiospermas, especialmente en grupos de plantas que han sufrido especiación rápida (Seehausen 2004).

Evolución mediada por polinizadores

La diversificación de las angiospermas ha atraído enormemente a biólogos evolucionistas desde que Darwin publicase sus estudios sobre la especialización floral en orquídeas (Darwin 1862). Se han propuesto varias hipótesis para explicar la enorme riqueza de especies de plantas con flor. Entre ellas, se ha sugerido que las interacciones con las distintas especies de polinizadores podrían ser la causa de la diversidad observada. Si el éxito reproductivo de una planta está ligado a la atracción de sus visitantes florales, los polinizadores podrían ejercer selección de caracteres florales (recompensa, forma, color, esencias o disposición). Esto podría dar lugar a especialización floral, aislamiento reproductivo y especiación (Grant 1949). Sin embargo, estudios empíricos sobre las interacciones

entre plantas y las comunidades de polinizadores han mostrado que la mayoría de los visitantes florales polinizan múltiples especies de plantas y que la mayoría de las plantas zoófilas son visitadas por múltiples especies de animales (Herrera 1996; Waser *et al.* 1996; Johnson & Steiner 2000; Strauss *et al.* 2005). Los polinizadores pueden variar mucho en su contribución al éxito reproductivo por su diferente frecuencia de visitas y/o eficacia en el transporte de polen. Pero para que el aislamiento floral diese lugar a especiación simpátrida los caracteres florales deberían ser suficientemente fuertes y estables para atraer a diferentes polinizadores casi instantáneamente. Por esta razón la especiación simpátrida mediada por polinizadores se ha considerado improbable (Coyne & Orr 2004).

En los últimos años se ha considerado otra hipótesis alternativa en la que la polinización biótica contribuiría a la diversificación de forma indirecta. Este escenario contempla que los linajes podrían aumentar la divergencia por medio del desplazamiento o refuerzo de caracteres florales (*Character-Displacement* model or *Reinforcement* model) (Armbruster & Muchhala 2009). En este caso el aislamiento floral sería favorecido por los polinizadores, pero tras una divergencia inicial provocada por otros factores. Esta hipótesis, que ha tenido mayor aceptación, considera que el papel de los polinizadores es secundario y plantea que la divergencia inicial se produciría por aislamiento ecogeográfico a lo que acompañaría una presión selectiva secundaria aplicada por la comunidad de polinizadores (Grant 1949; van der Niet *et al.* 2006; Armbruster & Muchhala 2009; Kay & Sargent 2009). De esta forma un conjunto de factores (y no solo los polinizadores) serían responsables de la diversificación de las plantas con flor.

A pesar de que, bajo las hipótesis de desplazamiento o refuerzo, los polinizadores no serían los promotores de la especiación en plantas, éstos si tendrían un papel fundamental en el desarrollo de formas florales. En muchos linajes de plantas se ha observado que los síndromes de polinización (o caracteres florales asociados con grupos funcionales de polinizadores) son lábiles desde el punto de vista evolutivo (revisados en Kay & Sargent 2009). Otros grupos de plantas no muestran diferentes síndromes florales, y sin embargo muestran divergencia floral asociada con la identidad de los polinizadores o su importancia relativa dentro de un mismo síndrome (Schemske & Horvitz 1984; Johnson & Steiner 1997). La inclusión de las filogenias en el estudio de las características florales ha sido el salto fundamental para reconstruir el historial evolutivo de la diversidad floral y comprobar si los diferentes caracteres han evolucionado numerosas veces en la misma dirección. Esto ha permitido comprobar si los polinizadores han tenido un papel relevante en la diferenciación de las especies a través del cambio de caracteres florales específicos (Herrera 1993; Hodges & Arnold 1994; Schemske & Bradshaw 1999; Galen & Cuba 2001; Gómez *et al.* 2006).

ESCENARIO ESPACIO-TEMPORAL DE LA TESIS DOCTORAL

CAMBIOS CLIMÁTICOS DEL CUATERNARIO

La conjunción entre procesos climáticos complejos y condiciones ambientales heterogéneas ha dado lugar a una excepcional diversidad de plantas y endemismos en la región mediterránea (Medail & Quezel 1997; Thompson 2005). La crisis de salinidad del messiniense (*Messinian Salinity Crisis*) (5.77-5.33 Ma), la aparición del clima mediterráneo c. 3.2 Ma, y las oscilaciones climáticas del Cuaternario c. 2.8 Ma son factores fundamentales para explicar la heterogeneidad de patrones evolutivos en la cuenca mediterránea (Thompson 2005). Las oscilaciones climáticas son, con toda probabilidad, determinantes para explicar el cambio en la distribución geográfica y los patrones de diversidad de las plantas que observamos hoy en día (Stebbins 1950; Comes & Kadereit 1998). En los últimos años los análisis paleoclimáticos han evidenciado el alcance de las oscilaciones glaciales-interglaciales del Cuaternario (e.g. Shackleton & Opdyke 1973; Petit *et al.* 1999). Tras el establecimiento de las glaciaciones, hace c. 0.7 Ma el clima se caracterizó por un aumento en la duración de los ciclos glaciales con picos de periodos fríos y secos cada c. 100.000 años interrumpidos por intervalos cortos de c. 15.000 años cálidos y húmedos (Webb & Bartlein 1992). El último periodo frío, que terminó hace c. 10.000 años, dio lugar a contracciones del área de distribución de las especies europeas a lo que siguió una expansión durante la retracción de los glaciales (Hewitt 1996, 2004). Sin embargo, nuevas estimaciones del clima del último periodo glacial, sugieren una compleja variación espacial de las temperaturas (Wu *et al.* 2007) y una importante diversidad de refugios glaciales y patrones de colonización dentro de la cuenca del Mediterráneo (Gómez & Lunt 2006; Médail & Diadema 2009; Feliner 2011).

Gracias a los estudios paleoclimáticos y paleoecológicos se han obtenido datos reveladores del efecto de las variaciones climáticas del Cuaternario en los movimientos de las especies. Sin embargo, el registro fósil está sesgado a favor de especies polinizadas por viento y presentes en zonas pantanosas o turberosas. Además, los fósiles tienen limitada resolución taxonómica y en ocasiones no son fáciles de asignar correctamente a un linaje evolutivo concreto. Por esta razón, los análisis moleculares pueden ser más informativos que el registro fósil para dilucidar el efecto del clima en la distribución y diversificación de los linajes. Sin embargo, una aproximación combinada con datos fósiles, moleculares y modelos de distribución de especies será la más adecuada para obtener resultados precisos y fiables.

Desde el desarrollo de las filogenias moleculares se han podido inferir patrones generales de colonización o refugios glaciales. Además hay un buen número de artículos que sugieren el origen

de numerosas especies de plantas durante las glaciaciones del Cuaternario, invocando un modelo de especiación alopátrida mediante aislamiento geográfico de las poblaciones (e.g. Vargas *et al.* 2009; Fiz-Palacios *et al.* 2010; Valente *et al.* 2010; Bell *et al.* 2012). Sin embargo, aún queda por confirmar si los cambios climáticos recientes tuvieron además un efecto positivo a nivel macroevolutivo (especiación vs. extición). Esta es una cuestión difícil de resolver debido a la imprecisión en la reconstrucción de eventos de extinción a partir de las filogenias moleculares (Rabosky 2010). Actualmente, el reto se centra en la búsqueda de múltiples marcadores moleculares de evolución rápida para estimar de forma precisa y creíble los tiempos de divergencia dentro del Cuaternario, y de esta forma poder buscar indicios de evolución rápida durante este periodo.

LA PENÍNSULA IBÉRICA DURANTE EL CUATERNARIO

Se han localizado dos centros principales de biodiversidad vegetal en la cuenca Mediterránea: un polo oriental que incluye Turquía y Grecia y un polo occidental que incluye la Península Ibérica y Marruecos (Medail & Quezel 1997). Dentro de la Península Ibérica encontramos zonas de una biodiversidad excepcional como son las cordilleras béticas, donde la endemicidad supera el 50% (Gomez-Campo *et al.* 1984). Las montañas de la Península Ibérica albergan una mayor riqueza de especies que las zonas bajas, principalmente por una mayor conservación histórica debida a un reducido uso antropogénico del terreno, un reducido efecto del clima durante la estación seca y principalmente por su función como refugios glaciales que permitió eventos de especiación durante los ciclos climáticos del Pleistoceno (Hewitt 1996; Comes & Kadereit 1998; Taberlet *et al.* 1998; Lobo *et al.* 2001; Médail & Diadema 2009). La heterogeneidad topográfica de la Península Ibérica, con numerosas cadenas montañosas con disposición este-oeste, ha jugado un papel fundamental en la distribución y evolución de los linajes de plantas permitiendo la supervivencia de las poblaciones mediante migraciones altitudinales en búsqueda de un clima favorable. Además, debido a su posición geográfica, la Península está bajo la influencia climática tanto del Océano Atlántico como del Mar Mediterráneo. Esto ha permitido la presencia de un amplio abanico de microclimas a lo largo de su territorio, y por lo tanto, la presencia de múltiples refugios glaciales durante el Cuaternario (Gómez & Lunt 2006).

Las montañas ibéricas deben de haber sido refugios dinámicos, donde diferentes microhábitats (zonas húmedas resguardadas y zonas secas más expuestas a distintas altitudes) han sido ocupados sucesivamente por distintas especies durante los ciclos glaciales. Además, el hecho de que la historia de muchas plantas ibéricas pueda trazarse hasta el periodo Terciario (e.g. Magri *et al.* 2007)

implica que la Península Ibérica albergaba suficiente heterogeneidad espacial como para permitir la estabilidad y persistencia durante periodos de mayor duración que el Cuaternario. Esta variedad topográfica y geológica debió favorecer el aislamiento de poblaciones, haciendo que poblaciones con poca capacidad de dispersión divergiesen en cortos periodos de tiempo (Petit *et al.* 2003). Las montañas del sur de la Península han jugado un papel determinante en la supervivencia de pequeñas poblaciones de herbáceas. Por ejemplo Sierra Nevada (3482 m), que representa el límite sur de la influencia glacial en Europa, estuvo cubierta de hielo solo por encima de c. 2500 m durante la última glaciación (Gómez-Ortiz *et al.* 1996). En esta cadena montañosa, amplias áreas quedaron libres de hielo permanente, lo que favoreció la persistencia y diversificación de especies de plantas alpinas (Kropf *et al.* 2006).

En la Península Ibérica se produjeron desplazamientos altitudinales siguiendo el modelo de expansión-contracción partiendo de los múltiples refugios glaciales presentes en las zonas montañosas. Como consecuencia se produjo un contacto secundario y la hibridación entre especies, lo cual debió ocurrir en zonas poco definidas y poco estables, siendo hoy en día muy difícil de identificar (Vargas 2003; Feliner 2011). La hibridación promovida por las oscilaciones climáticas parece particularmente relevante para los grupos de plantas de la Península donde diferentes hábitats podían encontrarse a cortas distancias en altitud o latitud y donde se minimizó la extinción de las poblaciones y genotipos debido al limitado efecto de los ciclos climáticos, si se comparan con su efecto en zonas más norteñas de Europa. Este panorama sugiere que los eventos de hibridación/introgresión, e incluso la especiación rápida mediada por hibridación, han podido ser procesos muy relevantes para explicar la excepcional diversidad encontrada en la Península Ibérica. Estos procesos podrían explicar la incertidumbre e incongruencia en las reconstrucciones filogenéticas de ciertos grupos de plantas (Albaladejo *et al.* 2005; Martín-Bravo *et al.* 2010; Vilatersana *et al.* 2010; Wilson & Hudson 2011). A pesar del probable efecto positivo de las variaciones climáticas del Cuaternario en la especiación, algunos estudios han documentado un efecto negativo debido a contracciones extremas del hábitat y extinciones locales (Willis & Niklas 2004). En este sentido las extinciones deben de haber sido importantes para especies termófilas que durante las épocas frías no pudieron refugiarse en las penínsulas del sur de Europa, así como para especies de climas fríos que con el aumento de las temperaturas no pudieron sobrevivir en los refugios del sur ni tampoco recolonizar el norte (Bennett *et al.* 1991).

GRUPO DE ESTUDIO

EL GÉNERO *LINARIA*

Linaria Mill. es el género más diverso de la tribu de las Antirrhineas (Fam. Plantaginaceae) con unas 150 especies (Sutton 1988) de distribución Paleártica, y con su máxima diversidad en la región Mediterránea. La evolución del género ha sido estudiada históricamente desde diversas disciplinas, tales como: macro-, micromorfología y anatomía comparada (Champagnat 1961; Valdés 1968; Viano 1978d; Sutton 1988; Juan *et al.* 1999a; Juan *et al.* 1999b), citogenética (Heitz 1927; Valdés 1969; Viano 1971, 1973), genética del desarrollo y epigenética (Cubas *et al.* 1999; Galego & Almeida 2007; Box *et al.* 2011), fitoquímica (Valdés 1970a; Handjieva *et al.* 1993; Nikolova-Damyanova *et al.* 1994; Beninger *et al.* 2009), interacciones ecológicas (Arnold 1982; Stout *et al.* 2000; Newman & Thomson 2005b, a; Sánchez-Lafuente 2007; Sánchez-Lafuente *et al.* 2011), biología reproductiva (Hill 1909; Bruun 1937; Valdés 1970c; Docherty 1982; McClay 1992; Valdés & Lifante 1996), hibridación (East 1933; Bruun 1937; Valdés 1970d; Viano 1978a), genética de poblaciones (Crawford & Elisens 2006; Segarra-Moragues & Mateu-Andres 2007), biogeografía/filogeografía (East 1933; Fernández-Mazuecos & Vargas 2011, 2013) y filogenia (Fernández-Mazuecos *et al.* 2013a; Fernández-Mazuecos *et al.* 2013b). Además, el género ha sido objeto de amplias revisiones taxonómicas y sistemáticas (Valdés 1970b; Viano 1978b, c; Sutton 1988; Sáez & Bernal 2009). En la revisión de Fernández-Mazuecos (2012) se recopila de forma detallada la bibliografía y el conocimiento previo sobre el género *Linaria*. Aquí nos centraremos en la información disponible sobre la sect. *Supinae*.

LA SECCIÓN *SUPINAE*

Antecedentes

Linaria se reconoció como entidad taxonómica independiente a finales del siglo XVII (Morison 1680; Tournefort 1700). Sin embargo, las especies de *Linaria* habían sido tradicionalmente incluidas dentro del género *Antirrhinum*, y así lo consideró Linneo (1753) en su *Species plantarum*. De esta forma *Linaria* no se pudo reconocer como género independiente hasta que Miller (1754) diferenció *Antirrhinum*, *Asarina* y *Linaria* y proporcionó una descripción post-linneana válida de estos géneros.

En un principio, la circunscripción del género *Linaria* era más amplia de la que consideramos hoy en día, ya que incluía todas aquellas especies próximas a *Antirrhinum* que mostraban un espolón en la corola. El género *Linaria sensu lato* quedó reconocido de forma independiente en los diversos

tratamientos taxonómicos posteriores a Miller (Moench 1794; Lamarck & De Candolle 1805; Du Mortier 1827; Chavannes 1833; Bentham 1846; Lange 1870; Boissier 1879). El género *Linaria sensu stricto* (tal y como lo conocemos hoy en día) se corresponde con la sect. *Linariastrum* de estos tratamientos taxonómicos mencionados. Los últimos autores subdividieron la sect. *Linariastrum* en distintos grupos. Sin embargo, estos tratamientos se caracterizaron por la falta de consenso en las subdivisiones taxonómicas de la sección (subsecciones o grupos) (Chavannes 1833, 5 subsecciones; Bentham 1846, 7 subsecciones; Lange 1870, 2 grupos; Boissier 1879, 2 grupos).

Posteriormente, Wettstein (1895) revisó de nuevo el género completo para *Die Natürligen Pflanzenfamilien* de Engler & Prantl, conservando casi exactamente la subdivisión de *Linaria sensu lato* adoptada por Bentham (1846). Sin embargo, Wettstein reconoció como géneros aparte las cuatro secciones que Chavannes había formado dentro de *Linaria sensu lato*, separando así los géneros *Chaenorrhinum, Cymbalaria*, *Elatinoides* (Chav.) Wettst. (=*Kickxia* Dumort.) y *Linaria*. Dentro del género *Linaria* (anterior sect. *Linariastrum* Chav.), Wettstein elevó todas las subsecciones que había propuesto Bentham (excepto una) a categoría de sección. Al igual que autores previos (Morison 1680; Lange 1870; Boissier 1879), Wettstein separó las seis secciones en dos grupos: uno formado por las secciones de semillas aladas y otro por las secciones de semillas ápteras. Sin embargo, estudios más recientes tendieron a considerar que la clasificación basada en el ala de las semillas era artificial debido a que este parece ser un carácter convergente, tal y como sugiere su anatomía no homóloga (Valdés 1970b; Sutton 1988). En los últimos trabajos se reconoce como más acertada la última clasificación completa del género hecha por Sutton (1988), que consta de 150 especies clasificadas en siete secciones: *Linaria*, *Pelisserianae* Valdés, *Supinae* (Benth.) Wettst., *Versicolores* (Benth.) Wettst., *Speciosae* (Benth.) Wettst., *Diffusae* (Benth.) Wettst., *Macrocentrum* D.A. Sutton. Además se ha considerado la posibilidad de que el género *Nuttallanthus* propuesto por Sutton sea una sección de *Linaria* (sect. *Lectoplectron*, Pennell 1935; Valdés 1970b). La sección más problemática sería la sect. *Diffusae* por la ausencia de sinapomorfías y las importantes diferencias entre las distintas especies (Valdés 1970b; Sutton 1988). La definición de subsecciones, así como de especies y subespecies, ha sido muy contradictoria en las últimas revisiones del género y particularmente en la sect. *Supinae* (Benth) Wettst, lo cual queda reflejado en las distintas, y en ocasiones, contradictorias delimitaciones taxonómicas y sistemáticas (véase Tabla Suplementaria 1). En los últimos tres tratamientos taxonómicos la sect. *Supinae* se ha subdividido en 2-3 subsecciones no coincidentes (Valdés 1970b: subsect. *Supinae*, subsect. *Amehystea* y subsect. *Saxatile*; Sutton 1988: subsect. *Supinae*, subsect. *Saxatile* y subsect. *Trimerocalyx*; Sáez & Bernal 2009: subsect. *Supinae* y subsect. *Saxatile*).

Características morfológicas

Se indican a continuación los caracteres morfológicos de la sect. *Supinae* del género *Linaria* modificado de Sutton (1988) y Sáez and Crespo (2005):

Hierbas anuales, bienales o perennes, glabras, pelosas o glandular-pubescentes. Tallos heteromorfos, raramente homomorfos. Tallos fértiles procumbentes, ascendentes o erectos. Los tallos estériles son numerosos y normalmente más cortos que los tallos fértiles. Brácteas reflejas –rara vez patentes-. Hojas homomorfas o heteromorfas, enteras, pinnatinervias, de lineares a suborbiculares, generalmente planas, en ocasiones canaliculadas o levemente revolutas en el margen, sésiles. Las hojas de los tallos fértiles verticiladas en verticilos de 3-6 (-8) abajo o en toda su longitud, a veces alternas arriba. Inflorescencia terminal en racimo, espiga o panícula, generalmente laxa. Brácteas opuestas, alternas o verticiladas, de lineares a suborbiculares, reducidas progresívamente hacia el ápice. Flores zigomorfas, pediceladas. Cáliz con sépalos subiguales o desiguales, con el sépalo superior más largo, libres excepto en la base. Corola personada, en ocasiones ± versicolor, de color blanco, amarillo, rojizo, violeta, rosa o lila, con frecuencia con venas más obscuras, exteriormente glabra o glandular-pubescente, papilosa en el interior, tubo ± cilíndrico, prolongado en la base en un espolón estrechamente cónico, agudo, recto o ± incurvado; labio superior erecto o erecto-patente, con 2 lóbulos profundamente separados, planos o ligeramente curvados; labio inferior reflejo, con 3 lóbulos, prolongado en un paladar que ocluye la boca del tubo. Androceo didínamo, con estambres inclusos; filamentos estaminales inferiores sin apéndice lateral en la base. Gineceo con ovario bilocular, estilo no dividido; estigma entero. Cápsula de oblonga a globosa, glabra o peloso-glandulosa hacia el ápice, truncada o ligeramente emarginada, lóculos iguales, dehiscentes por 3 valvas que llegan hasta la mitad de su longitud o hasta c. ¼ a cerca de su base. Semillas en disposición horizontal en las cápsulas, por lo general netamente comprimidas lateralmente, discoideas, plano-convexas o cóncavo-convexas, en ocasiones reniformes, rara vez subtrígonas; hilo ± hundido en el ala; margen por lo general con una ala prominente, rara vez con una cresta inconspicua; caras intermedias tuberculadas o lisas en ocasiones crestadas; pared periclinal de las células de la testa normalmente tabular, a veces papilosa.

Distribución y hábitat

Se considera que el centro de diversificación de la sect. *Supinae* ha sido la Península Ibérica debido al elevado número de especies encontradas, 40 de 44 según Sutton (1988). Las especies de la sect. *Supinae* habitan en lugares muy diversos incluyendo bordes de cultivo, dunas costeras, arenales, pedregales, claros de matorral, pastos, bosques o roquedos. En su conjunto ocupan un rango

altitudinal que va de los 0 m (*L. polygalyfolia*, *L. tursica, L. arenaria*) a los 3300 m (*L. glacialis, L. alpina*) sobre el nivel del mar. Ocupan sustratos arenosos, arcillosos, ácidos o básicos de forma específica o bien son indiferentes al sustrato. Las especies de la sect. *Supinae* tienen un amplio abanico de rangos de distribución, desde especies endémicas de distribución muy restringida (e.g. *L. glacialis*, *L. amoi*, *L. filicaulis*, *L. lilacina*, *L. platycalyx*) a especies con amplia distribución (*L. alpina*, *L. arvensis*, *L. micrantha*, *L. simplex*).

Biología reproductiva y polinización

Las complejas flores de *Linaria* sugieren una adaptación a la polinización entomófila. Aunque las especies de la sect. *Supinae* parecen ser predominantemente alógamas, se ha encontrado una amplia variabilidad en cuanto a los sistemas reproductivos. En ella se pueden encontrar especies completamente autógamas con flores muy pequeñas y poco llamativas, especies con autocompatibilidad ocasional y especies alógamas forzosas debido a un sistema de autoincompatibilidad gametofítica (Knuth 1909; Bruun 1937; Valdés 1970c; Docherty 1982).

La flor de las especies de la sect. *Supinae* se caracteriza por ser completamente ocluida (personada). En estas flores, el polinizador necesita ejercer una presión sobre el paladar para poder introducirse y así obtener la recompensa. En el proceso de entrada, el polinizador normalmente contacta con su tórax (*scutum*) las anteras y el estigma que se encuentran en la cara dorsal del interior de la flor. Este tipo de polinización (nototríbica) es la más común en todo el género (Macior 1967; Kampny 1995). La robustez de la corola, y por lo tanto, la fuerza requerida para su apertura es variable dentro de las especies de la sección. La mayoría de las especies tienen una robustez considerable y solo las abejas son capaces de ejercer suficiente presión, lo que limita la entrada de polinizadores menos efectivos como dípteros, lepidópteros y coleópteros. Sin embargo hay otras especies con corolas más débiles como *L. alpina*, que permiten la entrada de ciertos lepidópteros como la polilla diurna *Macroglossum stellatarum* (Knuth 1909).

Se han descrito algunas interacciones antagonistas con insectos que tienen un efecto negativo en el éxito reproductivo en las flores de *Linaria*. En la sect. *Supinae* se ha descrito herbivoría de las flores por parte de Ortópteros o larvas de Lepidópteros que afecta negativamente a la atracción de los polinizadores (Sánchez-Lafuente 2007). También es importante resaltar el efecto negativo de ciertos coleópteros de los géneros *Brachypterolus*, *Mecinus* o *Rhinusa* que se alimentan de las semillas de varias especies de *Linaria* (Jeanneret & Schroeder 1992; McClay 1992; Newman & Thomson 2005b), incluyendo especies de la sect. *Supinae* (observación personal).

Hibridación

Históricamente se han observado procesos de hibridación espontánea en la naturaleza y bajo experimentación controlada entre distintas especies de la sect. *Supinae*, así como entre algunas especies de la sect. *Supinae* y especies de otras secciones. Los híbridos naturales se han descrito como tales por presentar caracteres morfológicos intermedios entre ambas especies progenitoras. Sin embargo, resultados de hibridación experimental indican que el individuo híbrido puede ser similar a uno de los progenitores sin llegar a mostrar caracteres intermedios (Naudin 1862; Valdés 1970d). En la Tabla 1 se recopilan los distintos híbridos descritos, tanto naturales como experimentales, en los que uno o los dos progenitores pertenecen a la sect. *Supinae.* A pesar de la incertidumbre en la viabilidad de las semillas (Valdés 1970d), se observa que las especies de la sect. *Supinae* pueden formar frutos al cruzarse entre sí y con ciertas especies de la sect. *Linaria* y la sect. *Speciosae*. Sin embargo, muestran una producción de frutos casi nula cuando los cruzamientos se efectúan con especies de la sect. *Versicolores*. El alto grado de fertilidad entre especies de la sect. *Supinae* parece reflejar el escaso tiempo de divergencia ocurrido entre estas, lo cual se traduce en ausencia de incompatibilidades postcigóticas.

Tabla 1. Hibridación natural y experimental (exitosa y fallida) encontrada en la literatura, en la que algún progenitor es una especie de la sect. *Supinae*. En los híbridos experimentales la primera especie representa al progenitor femenino.

Individuo híbrido	**Referencia**
Híbrido natural	
sect. *Supinae x* sect. *Supinae*	
L. alpina x *L. supina* (*L.* x *rocheri* Fournier)	Fournier (1946); Chassagne (1957)
sect. *Supinae x* sect. *Linaria*	
L. arvensis x *L. vulgaris* (*L.* x *heribaudi* Camus)	Rouy (1909)
sect. *Linaria x* sect. *Supinae*	
L. vulgaris x *L. arvensis* (*L.* x *heribaudi* Camus)	Fournier (1946)
Híbrido experimental fértil (producción de frutos)	
sect. *Supinae x* sect. *Supinae*	
L. alpina x *L. supina*	(Valdés 1970d)
L. alpina x *L. amoi*	(Bruun 1937)
L. amoi x *L. tristis*	(Valdés 1970d)
L. amoi x *L. supina*	(Bruun 1937)
L. aeruginea x *L. amoi*	(Valdés 1970d)
L. amethystea x *L. saxatilis*	(Valdés 1970d)
L. caesia x *L. alpina*	(Valdés 1970d)
L. platycalyx x *L. tristis*	(Valdés 1970d)
L. saxatilis x *L. amethystea*	(Valdés 1970d)

L. saxatilis x *L. saturejoides*	(Valdés 1970d)
L. supina x *L. alpina*	(East 1933; Valdés 1970d)
L. supina x *L. anticaria*	(Valdés 1970d)
L. supina x *L. amoi*	(Bruun 1937)
L. trists x *L. amoi*	(Valdés 1970d)
L. tristis x *L. aeruginea*	(Valdés 1970d)
L. tristis x *L. anticaria*	(Valdés 1970d)
sect. *Versicolores* x sect. *Supinae*	
L. bipartita x *L. platycalyx*	(Valdés 1970d)
sect. *Linaria* x sect. *Supinae*	
L. vulgaris x *L. tristis*	(Valdés 1970d)
sect. *Speciosae* x sect. *Supinae*	
L. ventricosa x *L. tristis*	(Valdés 1970d)
Híbridación experimental fallida	
sect. *Supinae* x sect. *Supinae*	
L. alpina x *L. caesia*	(Valdés 1970d)
L. amoi x *L. aeruginea*	(Valdés 1970d)
L. caesia x *L. tristis*	(Valdés 1970d)
L. platycalyx x *L. anticaria*	(Valdés 1970d)
L. tristis x *L. platycalyx*	(Valdés 1970d)
L. tristis x *L. saturejoides*	(Valdés 1970d)
sect. *Supinae* x sect. *Diffusae*	
L. anticaria x *L. hirta*	(Valdés 1970d)
sect. *Supinae* x sect. *Versicolores*	
L. anticaria x *L. viscosa*	(Valdés 1970d)
L. anticaria x *L. spartea*	(Valdés 1970d)
L. latifolia x *L. algarviana*	(Viano 1978a)
L. latifolia x *L. bipartita*	(Viano 1978a)
L. latifolia x *L. gharbensis*	(Viano 1978a)
L. latifolia x *L. heterophylla*	(Viano 1978a)
L. latifolia x *L. maroccana*	(Viano 1978a)
L. latifolia x *L. pseudoviscosa*	(Viano 1978a)
L. latifolia x *L. spartea*	(Viano 1978a)
L. latifolia x *L. ventricosa*	(Viano 1978a)
L. latifolia x *L. viscosa*	(Viano 1978a)
L. amethystea x *L. algarviana*	Viano (1978a)
L. amethystea x *L. bipartita*	Viano (1978a)
L. amethystea x *L. gharbensis*	Viano (1978a)
L. amethystea x *L. heterophylla*	Viano (1978a)
L. amethystea x *L. maroccana*	Viano (1978a)
L. amethystea x *L. pseudoviscosa*	Viano (1978a)
L. amethystea x *L. spartea*	Viano (1978a)
L. amethystea x *L. viscosa*	Viano (1978a)
L. verticillata x *L. algarviana*	Viano (1978a)
L. verticillata x *L. bipartita*	Viano (1978a)
L. verticillata x *L. gharbensis*	Viano (1978a)
L. verticillata x *L. heterophylla*	Viano (1978a)
L. verticillata x *L. spartea*	Viano (1978a)
L. verticillata x *L. viscosa*	Viano (1978a)
sect. *Supinae* x sect. *Speciosae*	
L. tristis x *L. ventricosa*	(Valdés 1970d)

sect. *Supinae x* sect. *Linaria*	
L. caesia x *L. vulgaris*	(Valdés 1970d)
sect. *Versicolores x* sect. *Supinae*	
L. bipartita x *L. amethystea*	(Valdés 1970d)
L. viscosa x *L. platycalyx*	(Valdés 1970d)
L. viscosa x *L. tristis*	(Valdés 1970d)
L. viscosa x *L. amethystea*	(Valdés 1970d)

Conservación

Varios taxones de la sect. *Supinae* merecen una atención especial debido a su riesgo de desaparición derivado en mayor o menor grado de las actividades humanas. Se indican a continuación las especies o subespecies amenazadas, así como los principales peligros que afectan a sus poblaciones.

Linaria arenaria DC.

Esta especie está distribuida en poblaciones aisladas de la costa occidental de Francia y Galicia. En la Península Ibérica la especie está restringida a tres pequeñas poblaciones gallegas. Esta linaria habita arenales costeros asentados, los cuales están altamente amenazados por la alteración humana. Se ha observado que la actividad humana disminuye drásticamente la densidad poblacional de esta especie principalmente debido al pisoteo. Este taxon se incluye en el Atlas y Libro Rojo de la Flora Vascular Amenazada de España (Bañares *et al.* 2004) con categoría de en Peligro Crítico (CR).

Linaria oblongifolia subsp. *benitoi* (Fern. Casas) L. Sáez *et al.*

Taxon endémico del levante almeriense, restringido a poblaciones localizadas entre Mojácar y Carboneras, en zonas medias y bajas de la Sierra de la Cabrera, sobre suelos silíceos o arenosos. Su extensión de presencia es inferior a los 10 km^2 en poblaciones dispersas y poco numerosas. La destrucción y fragmentación de su hábitat por actividades turísticas y urbanísticas es su principal amenaza. Se le ha otorgado la categoría de en Peligro Crítico (CR) según el Atlas y Libro Rojo de la Flora Vascular Amenazada de España (Bañares *et al.* 2004). Sus poblaciones carecen de protección legal.

Linaria orbensis Carretero & Boira

Endemismo alicantino que ocupa suelos de cultivo pedregosos y de baja labranza, también en bordes de caminos y ribazos. La principal amenaza son las actividades agrarias y la urbanización. El

taxon está categorizado como En Peligro (EN) según el Atlas y Libro Rojo de la Flora Vascular Amenazada de España (Bañares *et al.* 2004).

Linaria polygalyfolia subsp. *aguillonensis* (García Mart.) Castrov. & Lago

Esta subespecie de *Linaria polygalifolia* vive de forma muy restringida en acantilados y arenales costeros de la costa de A Coruña. Sus tamaños poblacionales han podido ser subestimados debido a la difícil orografía de la costa gallega. Este taxon aparece en el Atlas y Libro rojo de la Flora Vascular Amenazada de España (Bañares *et al.* 2004) con categoría de En Peligro (EN).

Linaria polygalifolia subsp. *lamarckii* (Rouy) D.A. Sutton.

Esta subespecie es un endemismo de los sistemas dunares del suroeste de la Península Ibérica. Su riesgo de extinción se debe a la vulnerabilidad de su hábitat altamente perturbado por las edificaciones y el uso recreativo de estas zonas (Arregui & Hiraldo 2009). Este taxon está protegido legalmente en la Comunidad Andaluza y catalogado como Vulnerable por la Ley 8/2003. También se incluye tanto en el Atlas y Libro Rojo de la Flora Vascular Amenazada de España (Bañares *et al.* 2004) como en la Lista Roja Andaluza (Cabezudo 2005) con categoría de en Peligro Crítico (CR).

Otros taxones de la sect. *Supinae* se consideran con un menor grado de amenaza, es decir Vulnerable (VU) según la UICN (Bañares *et al.* 2004; Moreno 2008): *Linaria aeruginea* subsp. *pruinosa* (Sennen & Pau) Chater & Valdés Berm., *Linaria amoi* Campo ex. Amo, *Linaria depauperata* subsp. *hegelmaieri* (Lange) De la Torre et. al., *L. glacialis* Boiss., *L. huteri* Lange, *L. oligantha* subsp. *valentina* Sutton, *L. supina* subsp. *maritima* (DC.) M. Laínz y *L. tursica* Valdés & Cabezudo.

ESTRUCTURA DE LA MEMORIA DOCTORAL E HIPÓTESIS

Las especies de *Linaria* sect. *Supinae* constituyen un grupo muy adecuado para el estudio de la evolución debido la variabilidad observada en los sistemas reproductivos, requerimientos ecológicos, formas de vida, formas florales y de semillas. Esta diversidad de caracteres en especies con un supuesto origen común (monofilético), hacen que este grupo sea especialmente apropiado para estudiar los patrones evolutivos mediante análisis comparativos.

La presente memoria de Tesis doctoral presenta cinco manuscritos científicos enfocados a distintos niveles taxonómicos: nivel macroevolutivo (género *Linaria*, sect. *Supinae*) y microevolutivo (subsect. *Supinae* y especie (*L. glacialis*)). A pesar de que se asocia microevolución a patrones intraespecificos, aquí también se han incluido patrones a nivel de subsección ya que en ésta se incluyen especies de diversificación muy reciente (véase Manuscrito 4). De esta forma la taxonomía nos ha proporcionado un marco para generar distintas hipótesis de diferenciación y evolución en el grupo de estudio. El **objetivo general** de la presente tesis doctoral es desentrañar patrones evolutivos en *Linaria* sect. *Supinae* y descubrir las consecuencias evolutivas derivadas de las características intrínsecas de las especies, con especial énfasis en las estrategias reproductivas. El estudio se articula mediante análisis filogenéticos a partir de secuencias de ADN, que se complementan con análisis biogeográficos, filogeográficos, demográficos, de diversificación y morfométricos, reconstrucción de caracteres y asociación de caracteres en la filogenia. Estos análisis se complementan con una recopilación y obtención de datos morfológicos, ecológicos, de biología reproductiva y de sus polinizadores.

OBJETIVOS

El **objetivo básico** de la presente tesis es someter a test la hipótesis de monofilia de la sect. *Supinae* para comprobar si se trata o no de un grupo natural con un origen común del que han surgido patrones evolutivos divergentes. Una vez evaluada la monofilia de la sect. *Supinae* y su clasificación interna se procede a evaluar la siguiente **hipótesis general**: *Los patrones evolutivos de Linaria sect. Supinae a distintas escalas temporales y taxonómicas han estado determinados en gran medida por las estrategias reproductivas de las especies y el marco geográfico de aislamiento*. Para evaluar dicha hipótesis general se presentan los siguientes **objetivos particulares** abordados en cinco manuscritos:

Manuscrito 1.

- Reconstruir la filogenia molecular de *Linaria* con una región variable del ADN nuclear (*internal transcribed spacer*, ITS) en la que se incluye un muestreo extensivo del género, con el mayor número posible de especies de todas las secciones propuestas por Sutton (1988) y prestando especial atención a las especies de la sect. *Supinae.*
- Analizar las relaciones filogenéticas del género *Linaria* con respecto a los distintos linajes de la tribu Antirrhineae, incluyendo el género próximo *Nutallanthus*.
- Evaluar la monofilia de *Linaria*, así como la monofilia de las distintas secciones y subsecciones establecidas por Sutton (1988).
- Analizar el valor taxonómico del tipo de semilla (alada vs. áptera) para la clasificación interna del género *Linaria*.

Manuscrito 2.

- Reconstruir filogenias de la sect. *Supinae* (y un amplio grupo externo) utilizando la región ITS y varias regiones de ADN adicionales (un gen nuclear de copia simple AGT1 y dos marcadores del ADN plastidial: *trn*S-*trn*G y *rpl*32-*trn*L^{UAG}).
- Evaluar el papel que han tenido la hibridación y el *incomplete lineage sorting* en las señales filogenéticas.
- Identificar especies de la sect. *Supinae* con un posible origen híbrido.
- Reconstruir el árbol de especies (*Species tree*) de la sect. *Supinae* y someter a test distintas subsecciones reconocidas por autores previos mediante una aproximación multi-locus basada en la teoría de la coalescencia (*multispecies coalescent model*).
- Obtener una datación de la divergencia de los linajes principales de la sect. *Supinae*.
- Reconstruir un árbol de especies multietiqueta (*multilabelled species tree*) en el que se pueda representar la evolución reticulada de la sect. *Supinae*

Manuscrito 3.

- Estudiar la biología reproductiva de las especies de la sect. *Supinae*.
- Recopilar datos morfológicos y ecológicos de las especies de la sect. *Supinae*.
- Trazar los patrones de colonización a escala continental (Mediterráneo) y escala regional (Península Ibérica) mediante análisis biogeográficos y filogeográficos.
- Analizar la influencia de caracteres intrínsecos de las especies en los patrones de colonización y las tasas de diversificación (especiación y extinción) de la sect. *Supinae*.

Manuscrito 4.

- Estudiar la morfología floral de las especies de la subsect. *Supinae* mediante análisis de morfometría geométrica.
- Identificar la fauna polinizadora de estas especies.
- Evaluar el papel que han tenido los polinizadores actuales en la diferenciación de las formas florales.
- Trazar el cambio de las formas florales en la filogenia de la subsect. *Supinae* para detectar posibles eventos de convergencia morfológica.

Manuscrito 5.

- Realizar análisis de la estructura genética de una especie endémica de la zona alpina de Sierra Nevada (España) (*L. glacialis*) por medio de dos regiones plastidiales (*rpl*32-*trn*L^{UAG} y *rps*162F2-*trn*K^{UUU}) y una región nuclear (AGT1).
- Modelizar la distribución potencial actual de *L. glacialis* y proyectar dicha distribución a climas pasados y futuros.
- Evaluar la influencia de los cambios climáticos recientes en la distribución y diversidad genética de la especie.
- Proyectar la influencia de los cambios climáticos futuros derivados del calentamiento global en la distribución y diversidad genética de la especie.
- Discutir el papel que ha podido jugar la biología reproductiva en la supervivencia y la variación genética espacial de *L. glacialis*.

REFERENCIAS

Albaladejo RG, Aguilar JF, Aparicio A, Feliner GN (2005) Contrasting nuclear-plastidial phylogenetic patterns in the recently diverged Iberian *Phlomis crinita* and *P. lychnitis* lineages (Lamiaceae). *Taxon* **54**, 987-998.

Armbruster WS, Muchhala N (2009) Associations between floral specialization and species diversity: cause, effect, or correlation? *Evolutionary Ecology* **23**, 159-179.

Arnold RM (1982) Pollination, predation and seed set in *Linaria vulgaris* (Scrophulariaceae). *American Midland Naturalist* **107**, 360-369.

Arregui LP, Hiraldo CR (2009) La recuperación de *Linaria lamarckii*. *Conservación vegetal*, 16-17.

Ashman T-L, Knight TM, Steets JA, *et al.* (2004) Pollen Limitation of Plant Reproduction: Ecological and Evolutionary Causes and Consequences. *Ecology* **85**, 2408-2421.

Avise JC (1987) Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. *Annual review of ecology and systematics. Vol. 18*, 489-522.

Avise JC (2001) *Phylogeography. The History and Formation of Species* Harvard University Press, Cambridge, Massachusetts. London, UK.

Ayala FJ, Fitch WM, Clegg MT (2000) *Variation and Evolution in Plants and Microorganisms: Toward a New Synthesis 50 Years after Stebbins* The National Academies Press.

Baker HG (1955) Self-Compatibility and Establishment After 'Long-Distance' Dispersal. *Evolution* **9**, 347-349.

Baker HG (1966) The Evolution, Functioning and Breakdown of Heteromorphic Incompatibility Systems. I. The Plumbaginaceae. *Evolution* **20**, 349-368.

Baker HG (1967) Support for Baker's Law-As a Rule. *Evolution* **21**, 853-856.

Bañares A, Blanca G, Güemes J, Moreno J, Ortiz S (2004) *Atlas y Libro Rojo de la flora vascular amenazada de España* Ministerio de Medio Ambiente. Dirección General de Conservación de la Naturaleza., Madrid, Spain.

Barrett SC (2002) The evolution of plant sexual diversity. *Nature Reviews Genetics* **3**, 274-284.

Beaumont MA, Panchal M (2008) On the validity of nested clade phylogeographical analysis. *Molecular Ecology* **17**, 2563-2565.

Beerli P, Felsenstein J (2001) Maximum likelihood estimation of a migration matrix and effective population sizes in n subpopulations by using a coalescent approach. *Proceedings of the National Academy of Sciences* **98**, 4563-4568.

Bell CD, Mavrodiev E, Soltis P, *et al.* (2012) Rapid diversification of *Tragopogon* and ecological associates in Eurasia. *Journal of evolutionary biology* **25**, 2470-2480.

Beninger CW, Cloutier RR, Grodzinski B (2009) A comparison of antirrhinoside distribution in the organs of two related Plantaginaceae species with different reproductive strategies. *Journal of Chemical Ecology* **35**, 1363-1372.

Bennett KD, Tzedakis PC, Willis KJ (1991) Quaternary refugia of north European trees. *Journal of Biogeography* **18**, 103-115.

Bentham G (1846) Scrophulariaceae. In: *Prodromus Systematis Universalis Regni Vegetabilis* (ed. De Candolle A), pp. 168-598, Paris.

Boissier E (1879) *Flora orientalis. Vol. 4*, Geneva and Basel.

Box MS, Dodsworth S, Rudall PJ, Bateman RM, Glover BJ (2011) Characterization of *Linaria* KNOX genes suggests a role in petal-spur development. *The Plant Journal* **68**, 703-714.

Bruun HG (1937) Genetical notes on *Linaria*, I-II. *Hereditas* **22**, 295-400.

Cabezudo B (2005) *Lista Roja de la flora vascular de Andalucía* Junta de Andalucía, Consejería de Medio Ambiente.

Carstens BC, Richards CL (2007) Integrating coalescent and ecological niche modeling in comparative phylogeography. *Evolution* **61**, 1439-1454.

Champagnat M (1961) Recherches de morphologie descriptive et expérimentale sur le genre *Linaria*. *Annales des Sciences Naturelles. Botanique et Biologie Végétale* **12**, 1-170.

Charlesworth D, Charlesworth B (1995) Quantitative genetics in plants: the effect of the breeding system on genetic variability. *Evolution*, 911-920.

Chavannes E (1833) *Monographie des Antirrhinées* Paris et Lausanne.

Comes HP, Kadereit JW (1998) The effect of Quaternary climatic changes on plant distribution and evolution. *Trends in Plant Science* **3**, 432-438.

Coyne JA, Orr HA (2004) *Speciation* Sinauer Associates, Sunderland, Massachusetts U.S.A. .

Crawford PT, Elisens WJ (2006) Genetic variation and reproductive system among North American species of *Nuttallanthus* (Plantaginaceae). *American Journal of Botany* **93**, 582-591.

Cubas P, Vincent C, Coen E (1999) An epigenetic mutation responsible for natural variation in floral symmetry. *Nature* **401**, 157-161.

Darwin C (1859) *On the origin of species by means of natural selection* John Murray, London. UK.

Darwin C (1862) *On the various contrivances by which British and foreign orchids are fertilised by insects: and on the good effects of intercrossing* London: J. Murray.

Docherty Z (1982) Self-incompatibility in *Linaria*. *Heredity* **49**, 349-352.

Drummond AJ, Ho SYW, Phillips MJ, Rambaut A (2006) Relaxed Phylogenetics and Dating with Confidence. *PLoS Biol* **4**, e88.

Du Mortier BC (1827) *Florula belgica, operis majoris prodromus*, Staminacia.

East E (1933) Genetic observations on the genus *Linaria*. *Genetics* **18**, 324.

Ellstrand NC, Whitkus R, Rieseberg LH (1996) Distribution of spontaneous plant hybrids. *Proceedings of the National Academy of Sciences* **93**, 5090-5093.

Endler JA (1973) Gene Flow and Population Differentiation: Studies of clines suggest that differentiation along environmental gradients may be independent of gene flow. *Science* **179**, 243-250.

Faegri K, Van der Pijl L (1979) The principles of pollination ecology, 3rd rev. edn. *Pergamon Press, Oxford*.

Feliner GN (2011) Southern European glacial refugia: A tale of tales. *Taxon* **60**, 365-372.

Felsenstein J (1981) Evolutionary trees from DNA sequences: a maximum likelihood approach. *Journal of Molecular Evolution* **17**, 368-376.

Fernández-Mazuecos M (2012) *Evolución en el género Linaria: biogeografía, cambio morfológico y sistemática de Linaria sect. Versicolores*, Departamento de Biología. Facultad de Ciencias (Universidad Autónoma de Madrid).

Fernández-Mazuecos M, Blanco-Pastor JL, Gómez JM, Vargas P (2013a) Corolla morphology influences diversification rates in bifid toadflaxes (*Linaria* sect. *Versicolores*). *Annals of Botany* **112**, 1705-1722.

Fernández-Mazuecos M, Blanco-Pastor JL, Vargas P (2013b) A Phylogeny of Toadflaxes (*Linaria* Mill.) Based on Nuclear Internal Transcribed Spacer Sequences: Systematic and Evolutionary Consequences. *International Journal of Plant Sciences* **174**, 234-249.

Fernández-Mazuecos M, Vargas P (2011) Historical isolation *versus* recent long-distance connections between Europe and Africa in bifid toadflaxes (*Linaria* sect. *Versicolores*) *Plos One* **6**, e22234.

Fernández-Mazuecos M, Vargas P (2013) Congruence between distribution modelling and phylogeographical analyses reveals Quaternary survival of a toadflax species (*Linaria elegans*) in oceanic climate areas of a mountain ring range. *New Phytologist* **198**, 1274-1289.

Fisher RA (1941) Average excess and average effect of a gene substitution. *Annals of Eugenics* **11**, 53-63.

Fiz-Palacios O, Vargas P, Vila R, Papadopulos AST, Aldasoro JJ (2010) The uneven phylogeny and biogeography of *Erodium* (Geraniaceae): radiations in the Mediterranean and recent recurrent intercontinental colonization. *Annals of Botany* **106**, 871-884.

Fowler NL, Levin DA (1984) Ecological constraints on the establishment of a novel polyploid in competition with its diploid progenitor. *American Naturalist*, 703-711.

Galego L, Almeida J (2007) Molecular genetic basis of flower colour variegation in *Linaria*. *Genetical Research* **89**, 129-134.

Galen C, Cuba J (2001) Down the tube: pollinators, predators, and the evolution of flower shape in the alpine skypilot, *Polemonium viscosum*. *Evolution* **55**, 1963-1971.

Goldberg EE, Igić B (2012) Tempo and mode in plant breeding system evolution. *Evolution* **66**, 3701-3709.

Goldberg EE, Kohn JR, Lande R, *et al.* (2010) Species Selection Maintains Self-Incompatibility. *Science* **330**, 493-495.

Gomez-Campo C, Bermudez-De-Castro L, Cagiga MJ, Sánchez-Yelamo MD (1984) Endemism in the Iberian Peninsula and Balearic Islands. *Webbia* **38**, 709-714.

Gómez-Ortiz A, Schulte L, Salvador-Franch F (1996) Contribución al conocimiento de la deglaciación reciente y morfología asociada del Corral del Veleta (Sierra Nevada). *Cadernos do Laboratorio Xeolóxico de Laxe* **21**, 543-558.

Gómez A, Lunt D (2006) Refugia within refugia: patterns of phylogeographic concordance in the Iberian Peninsula. In: *Phylogeography of Southern European Refugia* (eds. Weiss S, Ferrand N), pp. 155-188. Springer, The Netherlands.

Gómez JM, Perfectti F, Camacho JPM (2006) Natural selection on *Erysimum mediohispanicum* flower shape: insights into the evolution of zygomorphy. *The American Naturalist* **168**, 531-545.

Grant V (1949) Pollination Systems as Isolating Mechanisms in Angiosperms. *Evolution* **3**, 82-97.

Grant V (1981) *Plant Speciation*, 2nd edn. Columbia University Press, New York.

Handjieva NV, Ilieva EI, Spassov SL, Popov SS, Duddeck H (1993) Iridoid glycosides from *Linaria* species. *Tetrahedron* **49**, 9261-9266.

He Q, Edwards DL, Knowles LL (2013) Integrative testing of how environments from the past to the present shape genetic structure across landscapes. *Evolution* **67**.

Heitz E (1927) Chromosomen und Gestalt bei *Antirrhinum* und verwandten Gattungen. *Planta* **4**, 392-410.

Heled J, Bryant D, Drummond A (2013) Simulating gene trees under the multispecies coalescent and time-dependent migration. *BMC Evolutionary Biology* **13**, 1-10.

Hennig W (1950) *Grundzüge einer Theorie der phylogenetischen Systematik* Deutscher Zentral-Verlag.

Herbert W (1847) On Hybridization Amongst Vegetables. *Journal of Horticultural Society of London* **2**.

Herrera C (1996) Floral Traits and Plant Adaptation to Insect Pollinators: A Devil's Advocate Approach. In: *Floral Biology* (eds. Lloyd D, Barrett SH), pp. 65-87. Springer US.

Herrera CM (1993) Selection on floral morphology and environmental determinants of fecundity in a hawk moth-pollinated violet. *Ecological Monographs* **63**, 251-275.

Hewitt GM (1996) Some genetic consequences of ice ages, and their role in divergence and speciation. *Biological Journal of the Linnean Society* **58**, 247-276.

Hewitt GM (2004) Genetic consequences of climatic oscillations in the Quaternary. *Philosophical Transactions of the Royal Society B: Biological Sciences* **359**, 183-195.

Hey J (2010) Isolation with Migration Models for More Than Two Populations. *Molecular Biology and Evolution* **27**, 905-920.

Hey J, Nielsen R (2007) Integration within the Felsenstein Equation for Improved Markov Chain Monte Carlo Methods in Population Genetics. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 2785-2790.

Hill EJ (1909) Pollination in *Linaria* with special reference to cleistogamy. *Botanical Gazette* **47**, 454-466.

Ho SYW, Shapiro B (2011) Skyline-plot methods for estimating demographic history from nucleotide sequences. *Molecular Ecology Resources* **11**, 423-434.

Hodges SA, Arnold ML (1994) Floral and ecological isolation between *Aquilegia formosa* and *Aquilegia pubescens*. *Proceedings of the National Academy of Sciences* **91**, 2493-2496.

Holsinger KE (2000) Reproductive systems and evolution in vascular plants. *Proceedings of the National Academy of Sciences* **97**, 7037-7042.

Jain S (1976) The evolution of inbreeding in plants. *Annual Review of Ecology and Systematics* **7**, 469-495.

Jeanneret P, Schroeder D (1992) Biology and host specificity of *Mecinus Janthinus* Germar (col.: Curculionidae), a candidate for the biological control of yellow and dalmatian toadflax, *Linaria Vulgaris* (l.) mill, and *Linaria Dalmatica* (l.) mill.(Scrophulariaceae) in North America. *Biocontrol Science and Technology* **2**, 25-34.

Johnson S, Steiner K (1997) Long-tongued fly pollination and evolution of floral spur length in the *Disa draconis* complex (Orchidaceae). *Evolution* **51**, 45-53.

Johnson SD, Steiner KE (2000) Generalization versus specialization in plant pollination systems. *Trends in Ecology & Evolution* **15**, 140-143.

Juan R, Fernandez I, Pastor J (1999a) Morphological and anatomical studies of *Linaria* species from South-west Spain: Fruits. *Annals of Botany* **84**, 21-31.

Juan R, Pastor J, Fernandez I (1999b) Morphological and anatomical studies of *Linaria* species from south-west Spain: Seeds. *Annals of Botany* **84**, 11-19.

Kampny CM (1995) Pollination and flower diversity in Scrophulariaceae. *The Botanical Review* **61**, 350-366.

Kay KM, Sargent RD (2009) The role of animal pollination in plant speciation: integrating ecology, geography, and genetics. *Annual Review of Ecology, Evolution, and Systematics* **40**, 637-656.

Kingman JFC (1982) The coalescent. *Stochastic Processes and their Applications* **13**, 235-248.

Knuth P (1909) *Handbook of Flower Pollination: Based Upon Hermann Müller's Work 'The Fertilisation of Flowers by Insects'* Clarendon Press, Oxford.

Kropf M, Comes HP, Kadereit JW (2006) Long-distance dispersal vs vicariance: the origin and genetic diversity of alpine plants in the Spanish Sierra Nevada. *New Phytologist* **172**, 169-184.

Kubatko LS (2009) Identifying Hybridization Events in the Presence of Coalescence via Model Selection. *Systematic Biology* **58**, 478-488.

Lamarck JB, De Candolle AP (1805) *Flore française*, Paris.

Lange J (1870) Scrophulariaceae. In: *Prodromus florae Hispanicae* (eds. Willkomm M, Lange J), pp. 546-620, Stuttgart.

Lemey P, Rambaut A, Drummond AJ, Suchard MA (2009) Bayesian phylogeography finds its roots. *PLoS Computational Biology* **5**.

Linnaeus C (1753) *Species plantarum. Vol. 2*, Stockholm.

Linnaeus C (1760) Disquisitio de sexu plantarum. *ab Academia Imperiali Scientiarum Petropolitana praemio ornata. Amenitates Acad* **10**, 100-131.

Lobo JM, Castro I, Moreno JC (2001) Spatial and environmental determinants of vascular plant species richness distribution in the Iberian Peninsula and Balearic Islands. *Biological Journal of the Linnean Society* **73**, 233-253.

Macior LW (1967) Pollen-foraging behavior of Bombus in relation to pollination of nototribic flowers. *American Journal of Botany* **54**, 359-364.

Magri D, Fineschi S, Bellarosa R, *et al.* (2007) The distribution of *Quercus suber* chloroplast haplotypes matches the palaeogeographical history of the western Mediterranean. *Molecular Ecology* **16**, 5259-5266.

Mallet J (2007) Hybrid speciation. *Nature* **446**, 279-283.

Martín-Bravo S, Valcárcel V, Vargas P, Luceño M (2010) Geographical speciation related to Pleistocene range shifts in the western mediterranean mountains (*Reseda* sect. *Glaucoreseda*, Resedaceae). *Taxon* **59**, 466-482.

Mayr E (1963) *Animal species and evolution* Harvard University Press, Cambrige, MA.

McClay A (1992) Effects of *Brachypterolus pulicarius* (L.)(Coleoptera: Nitidulidae) on flowering and seed production of common toadflax. *The Canadian Entomologist* **124**, 631-636.

Médail F, Diadema K (2009) Glacial refugia influence plant diversity patterns in the Mediterranean Basin. *Journal of Biogeography* **36**, 1333-1345.

Medail F, Quezel P (1997) Hot-Spots Analysis for Conservation of Plant Biodiversity in the Mediterranean Basin. *Annals of the Missouri Botanical Garden* **84**, 112-127.

Mendel G (1866) Experiment on plant hybrids (English translation). In: *The Origin of Genetics: A Mendel Source Book (1966)* (eds. Stern C, Sherwood ER). W. H. Freeman.

Miller P (1754) *The gardeners dictionary, 4th ed.*, London.

Moench C (1794) *Methodus plantas horti Marburgensis a staminun situ describendi*, Marburg. Germany.

Moreno J (2008) Lista Roja 2008 de la flora vascular española. Dirección General de Medio Natural y Política Forestal (Ministerio de Medio Ambiente, y Medio Rural y Marino, y Sociedad Española de Biología de la Conservación de Plantas), Madrid, 86 pp.

Morison R (1680) *Plantarum historiae universalis Oxoniensis. Vol. 2*, Oxford.

Müntzing A (1930) Outlines to a genetic monograph of the genus Galeopsis. *Hereditas* **13**, 185-341.

Naudin C (1862) *Nouvelles recherches sur l'hybridité dans les végétaux*.

Newman DA, Thomson JD (2005a) Effects of nectar robbing on nectar dynamics and bumblebee foraging strategies in *Linaria vulgaris* (Scrophulariaceae). *Oikos* **110**, 309-320.

Newman DA, Thomson JD (2005b) Interactions among nectar robbing, floral herbivory, and ant protection in *Linaria vulgaris*. *Oikos* **110**, 497-506.

Nikolova-Damyanova B, Ilieva E, Handjieva N, Bankova Y (1994) Quantitative thin layer chromatography of iridoid and flavonoid glucosides in species of *Linaria*. *Phytochemical Analysis* **5**, 38-40.

Pennell FW (1935) The Scrophulariaceae of eastern temperate North America. *The Academy of Natural Sciences of Philadelphia Monographs* **1**, 1-650.

Petit J-R, Jouzel J, Raynaud D, *et al.* (1999) Climate and atmospheric history of the past 420,000 years from the Vostok ice core, Antarctica. *Nature* **399**, 429-436.

Petit RJ, Aguinagalde I, de Beaulieu J-L, *et al.* (2003) Glacial Refugia: Hotspots But Not Melting Pots of Genetic Diversity. *Science* **300**, 1563-1565.

Posada D, Crandall K, Templeton A (2000) GeoDis: a program for the cladistic nested analysis of the geographical distribution of genetic haplotypes. *Molecular Ecology* **9**, 487-488.

Pybus OG, Rambaut A, Harvey PH (2000) An integrated framework for the inference of viral population history from reconstructed genealogies. *Genetics* **155**, 1429-1437.

Rabosky DL (2010) Extinction rates should not be estimated from molecular phylogenies. *Evolution* **64**, 1816-1824.

Richards AJ (1997) *Plant Breeding Systems*, 2nd edn. Chapman & Hall, London, UK.

Richards CL, Carstens BC, Lacey Knowles L (2007) Distribution modelling and statistical phylogeography: an integrative framework for generating and testing alternative biogeographical hypotheses. *Journal of Biogeography* **34**, 1833-1845.

Rieseberg LH (1997) Hybrid origins of plant species. *Annual Review of Ecology and Systematics* **28**, 359-389.

Rieseberg LH, Raymond O, Rosenthal DM, *et al.* (2003) Major ecological transitions in wild sunflowers facilitated by hybridization. *Science* **301**, 1211-1216.

Roberts HF (1929) *Plant hybridization before Mendel* Princeton University Press, Princeton, NJ.

Rodríguez DJ (1996) A Model for the Establishment of Polyploidy in Plants. *The American Naturalist* **147**, 33-46.

Ronquist F (1997) Dispersal-vicariance analysis: a new approach to the quantification of historical biogeography. *Systematic Biology* **46**, 195-203.

Ronquist F, Sanmartín I (2011) Phylogenetic Methods in Biogeography. *Annual Review of Ecology, Evolution, and Systematics* **42**, 441-464.

Sáez L, Bernal M (2009) *Linaria* Mill. In: *Flora Iberica 13* (eds. Castroviejo S, Herrero A, Benedí C, Rico E, Güemes J), pp. 232-324. CSIC, Madrid. Spain.

Sáez L, Crespo MB (2005) A taxonomic revision of the *Linaria verticillata* group (Antirrhineae, Scrophulariaceae). *Botanical Journal of the Linnean Society* **148**, 229-244.

Sánchez-Lafuente A (2007) Corolla herbivory, pollination success and fruit predation in complex flowers: An experimental study with *Linaria lilacina* (Scrophulariaceae). *Annals of Botany* **99**, 355-364.

Sánchez-Lafuente AM, Rodríguez-Gironés MA, Parra R (2011) Interaction frequency and per-interaction effects as predictors of total effects in plant–pollinator mutualisms: a case study with the self-incompatible herb *Linaria lilacina*. *Oecologia* **168**, 153-165.

Sanderson MJ (1997) A nonparametric approach to estimating divergence times in the absence of rate constancy. *Molecular Biology and Evolution* **14**, 1218-1231.

Schemske DW, Bradshaw H (1999) Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*). *Proceedings of the National Academy of Sciences* **96**, 11910-11915.

Schemske DW, Horvitz CC (1984) Variation among floral visitors in pollination ability: a precondition for mutualism specialization. *Science* **225**, 519-521.

Seehausen O (2004) Hybridization and adaptive radiation. *Trends in Ecology and Evolution* **19**, 198-207.

Segarra-Moragues JG, Mateu-Andres I (2007) Levels of allozyme diversity in closely related toadflaxes (*Linaria*, Plantaginaceae) and their correspondence with the breeding systems of the species. *Conservation Genetics* **8**, 373-383.

Shackleton NJ, Opdyke ND (1973) Oxygen isotope and palaeomagnetic stratigraphy of Equatorial Pacific core V28-238: Oxygen isotope temperatures and ice volumes on a 105 year and 106 year scale. *Quaternary Research* **3**, 39-55.

Stebbins GL (1950) *Variation and Evolution in Plants.* Columbia University Press, New York. USA.

Stebbins GL (1957) Self Fertilization and Population Variability in the Higher Plants. *The American Naturalist* **91**, 337-354.

Stebbins GL (1970) Adaptive Radiation of Reproductive Characteristics in Angiosperms, I: Pollination Mechanisms. *Annual Review of Ecology and Systematics* **1**, 307-326.

Stout JC, Allen JA, Goulson D (2000) Nectar robbing, forager efficiency and seed set: Bumblebees foraging on the self incompatible plant *Linaria vulgaris* (Scrophulariaceae). *Acta Oecologica* **21**, 277-283.

Strauss SY, Sahli H, Conner JK (2005) Toward a more trait-centered approach to diffuse (co) evolution. *New Phytologist* **165**, 81-90.

Sutton DA (1988) *A revision of the tribe Antirrhineae* Oxford University Press, Oxford.

Taberlet P, Fumagalli L, Wust-Saucy AG, Cosson JF (1998) Comparative phylogeography and postglacial colonization routes in Europe. *Molecular Ecology* **7**, 453-464.

Templeton AR (1998) Nested clade analyses of phylogeographic data: testing hypotheses about gene flow and population history. *Molecular Ecology* **7**, 381-397.

Thompson JD (2005) *Plant Evolution in the Mediterranean* Oxford University Press., New York. USA.

Tournefort JP (1700) *Institutiones rei herbariae*, Paris.

Valdés B (1968) Taxonomía experimental del género linaria: I. Germinación de las semillas y crecimiento de las plantas. *Botanica Complutensis* **1**, 131.

Valdés B (1969) Taxonomía experimental del género *Linaria* III. Cariología de algunas especies de *Linaria*, *Cymbalaria* y *Chaenorrhinum*. *Boletín de la Real Sociedad Española de Historia Natural (Biología)* **67**, 243-256.

Valdés B (1970a) Flavonoid pigments in flower and leaf of the genus *Linaria* (Scrophulariaceae). *Phytochemistry* **9**, 1253-1260.

Valdés B (1970b) Revisión de las especies europeas de *Linaria* con semillas aladas. *Anales de la Universidad Hispalense* **7**, 1-288.

Valdés B (1970c) Taxonomía experimental del género *Linaria* IV. Reproducción sexual: autogamia y polinización intraespecífica. *Bol. R. Soc. Española Hist. Nat. (Biol.)* **68**, 79-89.

Valdés B (1970d) Taxonomía experimental del género *Linaria* V. Hibridacion interespecifica. *Acta Phytotaxonomica Barcinonensia* **4**.

Valdés B, Lifante ZD (1996) Habitual autogamy in *Linaria tursica* Valdés et Cabezudo (Scrophulariaceae). *Flora* **191**, 329-333.

Valente LM, Savolainen V, Vargas P (2010) Unparalleled rates of species diversification in Europe. *Proceedings of the Royal Society B: Biological Sciences* **277**, 1489-1496.

van der Niet T, Johnson SD, Linder HP (2006) Macroevolutionary data suggest a role for reinforcement in pollination system shifts. *Evolution* **60**, 1596-1601.

Vargas P (2003) Molecular evidence for multiple diversification patterns of alpine plants in Mediterranean Europe. *Taxon* **52**, 463-476.

Vargas P, Carrió E, Guzmán B, Amat E, Güemes J (2009) A geographical pattern of *Antirrhinum* (Scrophulariaceae) speciation since the Pliocene based on plastid and nuclear DNA polymorphisms. *Journal of Biogeography* **36**, 1297-1312.

Viano J (1971) Contribution a l'etude caryosystematique du genre *Linaria*. *Caryologia* **24**, 183-201.

Viano J (1973) Resultats caryologiques de quelques especes de *Linaria* et *Chaenorrhinum* recoltees au sud de la Peninsule Iberique. *Bol. Soc. Brot., Supl* **47**, 323-331.

Viano J (1978a) Croisements experimentaux interspecifiques au sein du genre *Linaria*. *Caryologia* **31(4)**, 383-425.

Viano J (1978b) Les linaires à graines aptères du bassin méditerranéen occidental. 1. *Linaria* sect. *Versicolores*. *Candollea* **33**, 33-88.

Viano J (1978c) Les linaires à graines aptères du bassin méditerranéen occidental. 2. *Linaria* sect. *Elegantes*, *Bipunctatae*, *Diffusae*, *Speciosae*, *Repentes*. *Candollea* **33**, 209-267.

Viano J (1978d) *Recherches palynologiques sur le genre Linaria* Instituto Botanico Antonio Jose Cavanilles.

Vilatersana R, Garcia-Jacas N, Garnatje T, *et al.* (2010) Molecular phylogeny of the genus *Ptilostemon* (Compositae: Cardueae) and its relationships with *Cynara* and *Lamyropsis*. *Systematic Botany* **35**, 907-917.

Waser NM, Chittka L, Price MV, Williams NM, Ollerton J (1996) Generalization in Pollination Systems, and Why it Matters. *Ecology* **77**, 1043-1060.

Webb T, Bartlein P (1992) Global changes during the last 3 million years: climatic controls and biotic responses. *Annual Review of Ecology and Systematics* **23**, 141-173.

Wettstein R (1895) Scrophulariaceae. In: *Die natürligen pflanzenfamilien* (eds. Engler A, Prantl K), pp. 39-107, Lepzig.

Willis KJ, Niklas KJ (2004) The role of Quaternary environmental change in plant macroevolution: the exception or the rule? *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences* **359**, 159-172.

Wilson P, Thomson JD (1996) How Flowers Diverge? In: *Floral Biology: Studies on Floral Evolution in Animal-pollinated Plants* (eds. Lloyd DG, Barrett SCH), pp. 88-111. Springer.

Wilson Y, Hudson A (2011) The evolutionary history of *Antirrhinum* suggests that ancestral phenotype combinations survived repeated hybridizations. *Plant Journal* **66**, 1032-1043.

Winge O (1917) The chromosomes. *Their numbers and general importance. Carlsberg Lab Copenhagen CR trav* **13**, 131-175.

Wu H, Guiot J, Brewer S, Guo Z (2007) Climatic changes in Eurasia and Africa at the last glacial maximum and mid-Holocene: reconstruction from pollen data using inverse vegetation modelling. *Climate Dynamics* **29**, 211-229.

Wyatt R (1983) *Pollinator-plant interactions and the evolution of breeding systems.* Academic Press, Orlando. Florida.

Yang Z, Rannala B (1997) Bayesian phylogenetic inference using DNA sequences: a Markov chain Monte Carlo method. *Molecular Biology and Evolution* **14**, 717-724.

Yu Y, Barnett RM, Nakhleh L (2013) Parsimonious Inference of Hybridization in the Presence of Incomplete Lineage Sorting. *Systematic Biology* **62**, 738-751.

Tabla Suplementaria 1 – Clasificaciones previas de las especies de la sect. *Supinae* (Benth) Wettst incluidas en Sutton (1988). Entre corchetes se indica el nombre del taxon según Sutton.

Chavannes (1833)	Bentham (1846)
Sect. III – *Linariastrum* Chav.	Sect. III – *Linariastrum* Chav.
Grupo I	Subsect. I - Grandes Benth.
L. *latifolia* Desf.	L. *latifolia* Desf.
L. *thymifolia* DC.	
	Subsect IV – Arvenses Benth.
Grupo IV	L. *micrantha* (Cav.) Spreng.
L. *micrantha* Spreng.	L. *arvensis* (L.) Desf.
L. *arvensis* Desf.	L. *simplex* Willd. DC.
Grupo V	Subsect VI – *Supinae* Benth.
L. *tristis* Mill.	Grupo I – Semina disco laevi late marginata
L. *tristis* var. *tenuifolia* Chav. [L. *aeruginea* (Gouan) Cav.]	L. *lusitanica* (Vent.) Hoffmanns. & Link [L. *polygalifolia* subsp. *lamarckii* (Rouy) Sutton]
L. *supina* Desf.	L. *thymifolia* (Vahl) DC.
L. *supina* var. *pyrenaica* Duby [L. *supina* subsp. *supina*]	L. *marginata* Desf. [L. *aeruginea* subsp subsp. *aeruginea* (Gouan) Cav.]
L. *supina* var. *maritima* Duby [L. *supina* subsp. *supina*]	L. *tristis* (L.) Mill.
L. supina var. *glaucophylla* Hoffmanns [L. *supina* subsp. *supina*]	L. *platycalyx* Boiss.
L. *alpina* DC.	L. *burjatica* Turez.
L. alpina var. *erecta* Chav.	L. *caesia* (Pers.) DC
L. *arenaria* DC.	L. *supina* (L.) Chaz.
L. *saxatilis* Hoffmans & Link	fma. *pyrenaica* (Chav.) Duby [L. *supina* subsp. *supina*], fma. *maritima* (Chav.) Duby [L. *supina* subsp. *supina*]
L. saxatilis var. *stricta* Chav.	L. *satureioides* Boiss.
L. *fontanesiana* Chav. [similar a L. *oligantha* Lange según Sutton, status incierto]	L. *alpina* (L.) Mill.
L. polygonifolia Spreng. [L. *amethystea* subsp. *amethystea* (Vent.) Hoffmanns. & Link]	L. *glacialis* Boiss.
L. *broussonetii* (Chav.) [L. *amethystea* subsp. *broussonetii* (Poiret) Malato-Beliz]	
L. *amethystea* Hoffmanns. & Link	Grupo II – Semina marginata, disco tuberculoso
L. *glauca* Spreng.	L. *polygonifolia* (Poir.) Spreng. [L. *amethystea* subsp. *amethystea* (Vent.) Hoffmanns. & Link]
L. *glauca* var. *bipunctata*	L. *fontanesiana* Chav. [similar a L. *oligantha* Lange según Sutton, status incierto]
	L. *pauciflora* (Bonpl.) [L. *amethystea* subsp. *amethystea* (Vent.) Hoffmanns. & Link]
No clasificada	L. *amethystea* (Vent.) Hoffmanns. & Link
L. *caesia* DC	L. *broussonetii* (Chav.) [L. *amethystea* subsp. *broussonetii* (Poiret) Malato-Beliz]
	L. *diffusa* Hoffmanns. & Link
	Grupo III - Semina angusto marginata, disco laevi
	L. *glauca* (Spreng,) Cav.
	L. *saxatilis* (Hoffmanns. & Link)

L. *arenaria* DC
L. *candollei* Chav. [L. *arenaria* DC]

Subsect VII – *Diffusae*
Grupo I - Latifoliae
L. *flava* Desf. [L. oligantha Lange. subsp. oligantha]

No clasificada
L. *filifolia* Lag. [*L. bipunctata subsp. bipunctata* L. (Chaz)]

Valdés (1970)	Sutton (1988)	Sáez & Bernal (2009)– Especies Ibéricas
Sect. III – *Arvenses* (Benth.) Wettst. L. *arvensis* (L.) Desf. L. *simplex* (Willd.) DC. L. *micrantha* (Cav.) Hoffmanns. L. *munbyana* Boiss. & Reut. var. *munbyana* [L. *munbyana* Boiss. & Reut.), var. *pygmaea* (Sampaio) Sampaio [L. *munbyana* Boiss. & Reut.]	Sect. IV – *Supinae* (Benth.) Wettst. Subsect. I - *Supinae* Benth. L. *supina* (L.) Chaz subsp. *supina*, subsp. *duriaeana* Sutton L. *alpina* (L.) Mill. L. *faucicola* Leresche & Levier L. *caesia* (Pers.) F. Dietr. L. *thymifolia* (Vahl) DC. L. *polygalifolia* Hoffmanns. & Link subsp. *polygalifolia*, subsp. *lamarckii* (Rouy) Sutton L. *platicalyx* Boiss. L. *glacialis* Boiss. L. *tristis* (L.) Mill. subsp. *tristis*, subsp. *pectinata* (Pau & Font Quer) Maire, subsp. *marginata* (Desf.) Maire, subsp. *mesatlantica* Sutton, subsp. *lurida* (Ball) Maire L. *aeruginea* (Gouan) Cav. subsp. *aeruginea,* subsp. *nevadensis* (Boiss.) Sutton, subsp. *pruinosa* (Sennen & Pau) Chater & Valdés L. *amoi* Campo ex Amo L. *depauperata* Leresche ex Lange L. *anticaria* Boiss. & Reut. L. *verticillata* Boiss. L. *lilacina* Lange L. *tuberculata* Sutton L. *oblongifolia* (Boiss.) Boiss. & Reut.	Sect. IV – *Supinae* (Benth.) Wettst. Subsect. I - *Supinae* Benth. L. *supina* (L.) Chaz subsp. *supina*, subsp. *maritima* (Lam. & DC.) [L. *supina* subsp. *supina*] L. *caesia* (Pers.) F. Dietr. L. *polygalifolia* Hoffmanns. & Link subsp. *polygalifolia*, subsp. *lamarckii* (Rouy) Sutton, subsp. *aguillonensis* (García Mart.) Castrov. & Lago [L. *thymifolia* (Vahl) DC.] L. *aeruginea* (Gouan) Cav. subsp. *aeruginea*, subsp. *cardonica* (Font Quer) L. Sáez & M. Sainz [L. *aeruginea* subsp. *aeruginea*], subsp. *pruinosa* (Sennen & Pau) Chater & Valdés, subsp. *nevadensis* (Boiss.) Sutton L. *tristis* (L.) Mill. subsp. *tristis* L. *accitensis* L. Sáez [L. badalii Willk.] L. *verticillata* Boiss. subsp. *verticillata* [L. *lilacina* Lange], subsp. *lilacina* (Lange) L. Sáez & M. B. Crespo [L. *lilacina* Lange], subsp. *cuartanensis* (Degen & Hervier) L. Sáez & M.B. Crespo [confundido con L. *anticaria* Boiss. & Reut., L. *lilacina* Lange y L. *tristis* subsp. tristis], subsp. *anticaria* (Boiss. & Reut.) L. Saez & M.B. Crespo [L. *anticaria* Boiss. & Reut.] L. *amoi* Campo ex Amo L. *depauperata* Leresche ex Lange
Sect. IV – *Supinae* (Benth.) Wettst. Subsect. *Supinae* Valdés. Grupo I L. *supina* (L.) Mill. L. *oblongifolia* (Boiss.) Boiss. & Reut. subsp. *oblongifolia*, subsp. *haenseleri* (Boiss. & Reut.) Valdés L. *caesia* (Pers.) DC var. *caesia* [L. *caesia* (Pers.) F. Dietr.], var. *decumbens* Lange [L. *poligalifolia* subsp. *polygalifolia*] L. *lamarckii* Rouy [L. *polygalifolia* subsp. *lamarckii* (Rouy) Sutton] L. *tristis* (L.) Mill. L. *aeruginea* (Gouan) Cav. var. *aeruginea* [L. *aeruginea* subsp. *aeruginea*], var. *nevadensis* (Boiss.) Valdés [L. *aeruginea* subsp. *nevadensis* (Boiss.) Sutton], var. *atrofusca* (Rouy) Sampaio [L. *aeruginea* subsp. *aeruginea*], var. *pruinosa* Sennen & Pau [L. *aeruginea* subsp. *pruinosa*		

(Sennen & Pau) Chater & Valdés)
L. *amoi* Campo ex Amo
L. *depauperata* Leresche ex Lange
var. *depauperata* [L. *depauperata* Leresche ex Lange], var. *hegelmaieri* (Lange) Willk. [L. *depauperata* Leresche ex Lange]
L. *badalii* Willk.
fma. *badalii* [L. *badalii* Willk.], fma. *odoratissima* (Bubani) Valdés [L. *badalii* Willk.]
L. *propinqua* Boiss. & Reut.
L. *glauca* (L.) Chaz.
subsp. *glauca*, subsp. *aragonensis* (Lange) Valdés [L. *oblongifolia* subsp. *aragonensis* (Lange) Sutton], subsp. *bubani* (Font Quer) Valdés [L. *bubanii* Font Quer]
L. *diffusa* Hoffmans & Link
L. *coutinhoi* Valdés [L. *intricata* Coincy]

Grupo II
L. *anticaria* Boiss. & Reut.
var. *anticaria* [L. *anticaria* Boiss. & Reut.], var. *angustifolia* Boiss. & Reut. [L. *anticaria* Boiss. & Reut.], var. *cuartanensis* (Deg. & Herb.) Deg. & Herb. [L. *verticillata* Boiss.]
L. *verticillata* Boiss.
L. *lilacina* Lange

Grupo III
L. *alpina* (L.) Mill.
var. *alpina* fma. *alpina* [L. *alpina* (L.) Mill.] , var. *alpina* fma. *concolor* (Bruhin) Hegi [L. *alpina* (L.) Mill.], var. *alpina* fma. *flava* (Gremli) Hegi [L. *alpina* (L.) Mill.], var. *alpina* fma. *rosea* (Ronninger) Hegi [L. *alpina* (L.) Mill.], var. *alpina* fma. *pilosa* (Fouc.) Valdés [L. *alpina* (L.) Mill.], var. *erecta* Chav. [L. *alpina* (L.) Mill.]
L. *filicaulis* Boiss. ex Leresche & Levier
L. *faucicola* Levier & Leresche [L. filicaulis Boiss.]

Grupo IV (Grupo artificial)
L. *ricardoi* Coutinho
subsp. *oblongifolia*, subsp. *haenseleri* (Boiss. & Reut.) Valdés, subsp. *aragonensis* (Lange) Sutton
L. *satureioides* Boiss.
L. *ricardoi* Cout.
L. *latifolia* Desf.
L. *glauca* (L.) Chaz.
subsp. *glauca*, subsp. *olcadium* Valdés & D. A. Webb
L. *badalii* Willk.
L. *propinqua* Boiss. & Reut.
L. *bubanii* Font Quer
L. *filicaulis* Boiss.
L. *amethystea* (Vent.) Hoffmanns. & Link
subsp. *amethystea*, subsp. *multipunctata* (Brot.) Chater & Webb, subsp. *ignescens* (Kunze) Sutton, subsp. *broussonetii* (Poiret) Malato-Beliz
L. *munbyana* Boiss. & Reut.
L. *tarhunensis* Pamp.
L. *fallax* G. Barratte
L. *arvensis* (L.) Desf.
L. *simplex* Willd. ex Desf.
L. *micrantha* (Cav.) Hoffmanns. & Link

Subsect. II - Saxatile Valdés
L. *saxatilis* (L.) Chaz.
L. *arenaria* DC
L. *oligantha* Lange
subsp. *oligantha*, subsp. *valentina* Sutton
L. *huteri* Lange
L. *ficalhoana* Rouy
L. *bipunctata* (L.) Chaz.
subsp. *bipunctata*, subsp. *glutinosa* (Hoffmanns. & Link) Sutton
L. *intricata* Coincy
L. *coutinhoi* Valdés
L. *diffusa* Hoffmanns. & Link
L. *atlántica* Boiss. & Reut.
L. *tursica* Valdés & Cabezudo

Subsect. III – Trimerocalyx (Murb) Sutton
L. *paradoxa* Murb.
subsp. *depauperata* [L. *depauperata* Leresche ex Lange], subsp. *ilergabona* (M. B. Crespo & Arán) L. Sáez [L. *depauperata* Leresche ex Lange], subsp. *hegelmaieri* (Lange) De la Torre, Alcaraz & M.B. Crespo [L. *depauperata* Leresche ex Lange]
L. *orbensis* Carretero & Boira [desconocida para Sutton]
L. *saturejoides* Boiss.
subsp. *saturejoides* [L. *satureioides* Boiss.], subsp. *angustealata* (Wilmott) Malag. [L. *satureioides* Boiss.],
L. *oblongifolia* (Boiss.) Boiss. & Reut.
subsp. *oblongifolia*, subsp. *haenseleri* (Boiss. & Reut.) Valdés, subsp. *aragonensis* (Lange) Sutton, subsp. benitoi (Fern. Casas) L. Sáez, M.B. Crespo [L. *tuberculata* Sutton]
L. *glacialis* Boiss.
L. *glauca* (L.) Chaz.
subsp. *glauca*, subsp. *olcadium* Valdés & D. A. Webb
L. *bubanii* Font Quer
L. *badalii* Loscos [L. *badalii* Willk.]
L. *propinqua* Boiss. & Reut.
L. *alpina* (L.) Mill
subsp. alpina [L. *alpina* (L.) Mill.], subsp. *filicaulis* (Boiss. ex Leresche & Levier) M. Laínz [L. *alpina* (L.) Mill.]
L. *platycalyx* Boiss.
L. *ricardoi* Cout.
L. *latifolia* Desf.
L. *amethystea* (Vent.) Hoffmanns. & Link
subsp. *amethystea*, subsp. *multipunctata* (Brot.) Chater & Webb, subsp. *ignescens* (Kunze) Sutton
L. *munbyana* Boiss. & Reut.
L. *arvensis* (L.) Desf.
L. *simplex* Willd. ex Desf.
L. *micrantha* (Cav.) Hoffmanns. & Link

Subsect. II - Saxatile Valdés
L. *saxatilis* (L.) Chaz.
L. *arenaria* DC
L. *oligantha* Lange
subsp. *oligantha*, subsp. *valentina* Sutton
L. *huteri* Lange

L. *glacialis* Boiss.
L. *platycalyx* Boiss.
L. *thymifolia* (Vahl)
L. *saturejoides* Boiss. [L. *satureioides* Boiss.]
var. *saturejoides* [L. *satureioides* Boiss.], var. *angustealata* (Wilmott) Valdés [L. *satureioides* Boiss.]

Subsect. Amethystea Valdés
L. *amethystea* (Lam.) Hoffmanns. & Link
var. *amethystea* fma. *amethystea* [L. *amethystea* subsp. *amethystea*], var. *amethystea* fma. *broussonetii* (Poir.) Valdés [L. *amethystea* subsp. *broussonetii* (Poiret) Malato-Beliz], var. *albiflora* Boiss. [L. *amethystea* subsp. *amethystea*]
L. *multipunctata* (Brot.) Hoffmans & Link [L. *amethystea* subsp. *multipunctata* (Brot.) Chater & Webb]

Subsect Saxatile Valdés
L. *saxatilis* (L.) Chaz.
var. *saxatilis* [L. *saxatilis* (L.) Chaz.], var. *glabrescens* (Lange) Rouy [L. *saxatilis* (L.) Chaz.], var. *glutinosa* (Hoffmans & Link) Rouy [L. *bipunctata* subsp. *glutinosa* (Hoffmanns. & Link) Sutton], var. *minor* (Lange) Sampaio [L. *saxatilis* (L.) Chaz.]

L. *amoris* Pau [L. *intricata* Coincy]
L. *arenaria* DC

L. *bipunctata* (L.) Chaz.
subsp. *bipunctata*, subsp. *glutinosa* (Hoffmanns. & Link) Sutton
L. *intricata* Coincy
L. *diffusa Hoffmanns*. & Link
L. *tursica* Valdés & Cabezudo

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A PHYLOGENY OF TOADFLAXES (*LINARIA* MILL.) BASED ON NUCLEAR INTERNAL TRANSCRIBED SPACER SEQUENCES: SYSTEMATIC AND EVOLUTIONARY CONSEQUENCES

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Premise of research. Toadflaxes (*Linaria* Mill., ~150 spp. from the Palearctic region) constitute the largest genus of the snapdragon lineage (tribe Antirrhineae). Here we provide the first extensive phylogenetic testing of systematic and evolutionary hypotheses about toadflaxes.

Methodology. Internal transcribed spacer (ITS) sequences were obtained for 94 species representing all sections of *Linaria* recognized by recent taxonomic treatments, as well as three species of the morphologically related American genus *Nuttallanthus*. In addition, 71 sequences representing the remaining 26 genera of Antirrhineae were gathered to test the monophyly of toadflaxes. Phylogenetic analyses were conducted using Bayesian inference and maximum likelihood. Evaluation of alternative topologies was assessed by means of Bayes factor analyses.

Pivotal results. *Linaria* and *Nuttallanthus* constituted a monophyletic group within the Antirrhineae. *Linaria* was recovered as a paraphyletic group with *Nuttallanthus* nested within it. Six major clades were recognized within the *Linaria-Nuttallanthus* clade. The seed wing, a structure that has been extensively used in systematic treatments, appears to be a homoplasious character in *Linaria*.

Conclusions. The circumscription of *Nuttallanthus* within *Linaria* is suggested to preserve the monophyly of the latter genus. Three sections of *Linaria* (*Macrocentrum*, *Pelisserianae*, and *Versicolores*) that are well defined by distinct morphological traits are also supported as natural groups, while monophyly of the remaining sections (*Supinae*, *Linaria*, *Speciosae*, and *Diffusae*) is unsupported by our results. Habit, inflorescence, and flower morphology, coupled with seed morphology, are revealed as the key characters in the evolution of toadflaxes.

Keywords: *Linaria*, *Nuttallanthus*, toadflax, seed evolution, phylogeny, internal transcribed spacer.

Online enhancement: appendix figure.

Introduction

One hundred fifty species of toadflaxes (*Linaria* Mill.; see examples in fig. 1) were proposed in the last taxonomic treatment of the tribe Antirrhineae (Sutton 1988). *Linaria* constitutes the largest of the 28 genera of this tribe and displays the key flower characteristics of *Antirrhinum*, a model group for plant developmental and evolutionary research (Schwarz-Sommer et al. 2003). The personate, spurred, usually occluded corolla of *Linaria* has also attracted attention as a model to understand the development and evolution of floral symmetry (Cubas et al. 1999), flower color (Galego and Almeida 2007), and nectar spurs (Box et al. 2011). Active research is also being undertaken on reproductive strategies and pollination (Sánchez-Lafuente et al. 2011), phytochemistry (Beninger et al. 2009), biogeography (Fernández-Mazuecos and Vargas 2011), and invasion biology (Sing and Peterson 2011). Despite this research attention, a well-supported evolutionary framework for *Linaria* research is still lacking.

Linaria was recognized as a taxonomic entity as early as the time of pre-Linnaean botanists (Morison 1680; Tournefort 1700). Linnaeus (1753) included previously recognized *Linaria* species within his genus *Antirrhinum*. However, *Linaria* was shortly after accepted as a distinct genus by Miller (1754), who provided the first valid description of the genus. Early authors generally considered the genus *Linaria* in a wide sense, including all those species related to *Antirrhinum* that display a spurred corolla (Lamarck and De Candolle 1805; Chavannes 1833; Bentham 1846). Chavannes (1833) delimited four sections within *Linaria* (*Chaenorhinum*, *Cymbalaria*, *Elatinoides*, and *Linariastrum*), which were later separated as distinct genera by Wettstein (1895), giving rise to the current circumscription of *Linaria* (formerly sect. *Linariastrum*). Only species with entire, sessile leaves and terminal, racemose inflorescences remained under this genus. This view has been adopted by all recent taxonomic treatments (Rothmaler 1943; Valdés 1970; Sutton 1988; Sáez and Bernal 2009) and has been supported by molecular phylogenetics (Ghebrehiwet et al. 2000; Vargas et al. 2004).

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Fig. 1 Representatives of *Linaria* and *Nuttallanthus*. *Linaria* sect. *Linaria*: *A*, *L. vulgaris*; *B*, *L. meyeri*; *C*, *L. japonica*. *Linaria* sect. *Speciosae*: *D*, *L. dalmatica*; *E*, *L. peloponnesiaca*; *F*, *L. repens*. *Linaria* sect. *Diffusae*: *G*, *L. albifrons*; *H*, *L. triphylla*; *I*, *L. haelava*; *J*, *L. hirta*. *Linaria* sect. *Supinae*: *K*, *L. latifolia*; *L*, *L. amethystea*; *M*, *L. depauperata*; *N*, *L. arvensis*; *O*, *L. saxatilis*; *P*, *L. tursica*. *Linaria* sect. *Pelisserianae*: *Q*, *L. triornithophora*; *R*, *L. pelisseriana*. *Linaria* sect. *Versicolores*: *S*, *L. viscosa*; *T*, *L. bipartita*; *U*, *L. clementei*; *V*, *L. nigricans*; *W*, *L. elegans*. *Linaria* sect. *Macrocentrum*: *X*, *L. chalepensis*; *Y*, *L. armeniaca*. *Nuttallanthus*: *Z*, *Z′*, *N. texanus*. Photos by M. Luceño (*A*, *D–F*, *Q*, *R*), O. Fragman-Sapir (*B*, *G–I*, *X*, *Y*), N. V. Kurzenko (*C*), J. Ramírez (*J–L*, *U*), J. L. Blanco-Pastor (*M–P*), J. Quiles (*S*, *T*, *W*), P. Vargas (*V*), and G. D. Carr (*Z*, *Z′*).

Subdivision of *Linaria* (in its currently accepted sense) has historically followed two main trends (reviewed by Valdés 1970; Sutton 1988). A first subdivision into two groups has been based on the presence or absence of an encircling wing in seeds (fig. 2). This approach seems to date back to Morison (1680) and was widely followed by later authors, but it has rarely been reflected in formal infrageneric taxa (Lange 1870; Wettstein 1895; Valdés 1970). Dumortier (1827) did distinguish the sections *Leontorrhinum* (winged seeds) and *Lycorrhinum* (wingless seeds), while Boissier (1879) called these groups *Discoideae* and *Oblongae*, respectively. Viano (1978*a*, 1978*b*) hypothesized that species with winged and wingless seeds constitute two sister evolutionary lineages. However, despite being of practical value, this two-partite classification has been considered artificial by Valdés (1970) and Sutton (1988) on the basis of the nonhomologous anatomy of seed wings across the genus.

A second, multipartite approach to *Linaria* subdivision has been based on a wider range of vegetative and reproductive traits. This strategy dates back to Chavannes (1833), who recognized five subdivisions within the current circumscription of *Linaria*. These groups were named and reorganized by Bentham (1846). Largely following Bentham's approach, Wettstein (1895) recognized six sections within *Linaria*, which constitute the base of subsequent classifications by Valdés (1970), Viano (1978*a*, 1978*b*), and Sutton (1988; table 1). Sutton's classification (table 2) is widely accepted today and includes 150 species classified in seven sections: *Linaria* (45 spp.), *Supinae* (44 spp.), *Pelisserianae* (2 spp.), *Versicolores* (21 spp.), *Speciosae* (19 spp.), *Diffusae* (17 spp.), and *Macrocentrum* (2 spp.). The first three sections include species with discoid, usually winged seeds, while the latter four contain species with nondiscoid, wingless seeds.

DNA sequences of eight species representing the seven sections of *Linaria* formed a monophyletic group in a previous phylogeny of the tribe Antirrhineae based on the nuclear internal transcribed spacer (ITS) region (Vargas et al. 2004). Naturalness of sections is, however, considered doubtful at least in some cases (Valdés 1970; Sutton 1988), and it has not been assessed in a molecular phylogenetic framework to date.

Linaria is basically a Palearctic genus. It has its diversity center in the Mediterranean region, where all seven sections of Sutton's classification are present. Only sect. *Linaria* has a wider range that extends over most of Eurasia and reaches the Japanese archipelago. The few toadflax species (with wingless seeds) native to the New World have historically been circumscribed in different genera. Because of the lack of a well-developed palate, they were included in *Anarrhinum* by Desfontaines (1798). More commonly, they have been included in *Linaria*, as part of sect. *Versicolores* (Bentham 1846; Wettstein 1895) or as the distinct sect. *Lectoplectron* (Pennell 1935; Valdés 1970). Finally, Sutton (1988) transferred these three North American and one South American species to his new genus *Nuttallanthus*, on the basis of flower and seed traits. Molecular phylogenies of Antirrhineae (Ghebrehiwet et al. 2000; Vargas et al. 2004) have not included *Nuttallanthus* accessions to date, and therefore the status of this genus as a distinct evolutionary lineage and its relationships within the tribe Antirrhineae remain unclear.

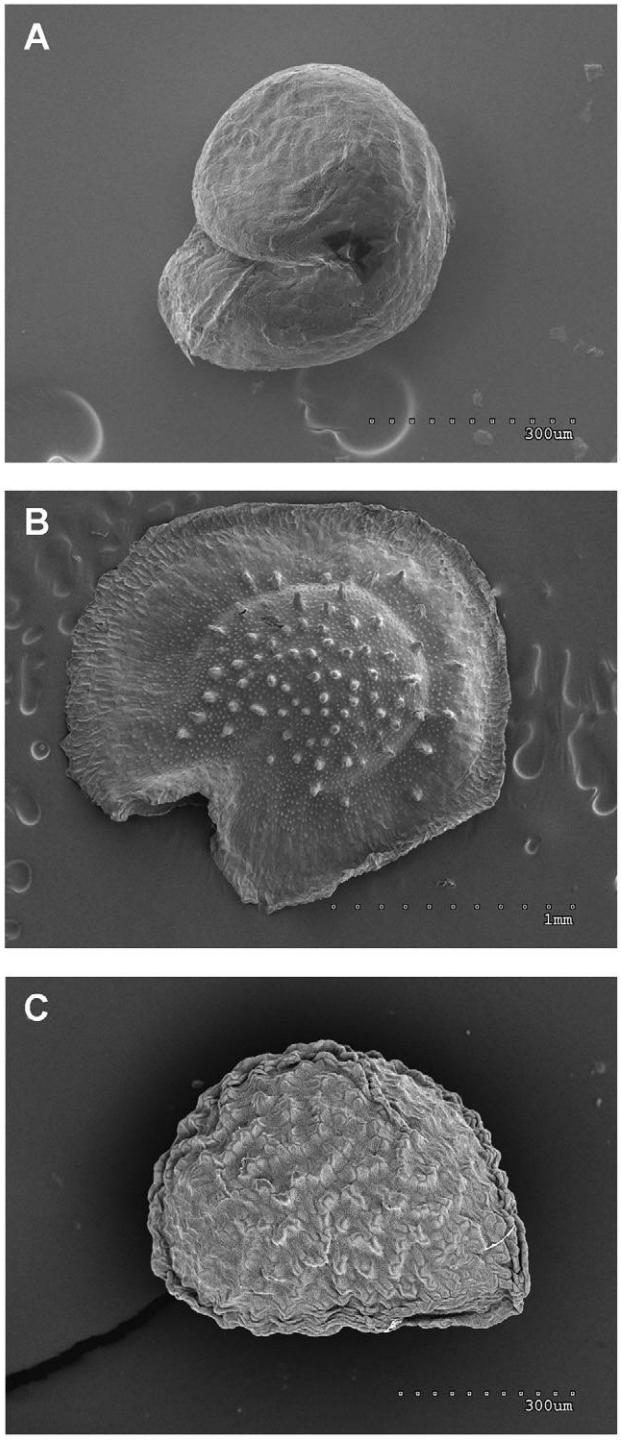


Fig. 2 Examples of *Linaria* seeds belonging to the three major morphological types. *A*, No wing (*L. tursica*). *B*, Wing (*L. simplex*). *C*, Marginal ridge (*L. huteri*). SEM photos by Y. Ruiz.

Here we present the first phylogenetic hypothesis of *Linaria* and *Nuttallanthus* based on nuclear ribosomal DNA sequences. A worldwide sampling of ITS sequences of all Antirrhineae genera, including a wide sample of *Linaria* species and three species of *Nuttallanthus*, has been performed to

Table 1

Historical Overview of *Linaria* and *Nuttallanthus* Classification

Sutton (1988), Sáez and Bernal (2009), and this study	Bentham (1846)	Wettstein (1895)	Valdés (1970) and Viano (1978*a*, 1978*b*)
Linaria Mill.	*Linaria* sect. *Linariastrum* Chav.	*Linaria* Juss.	*Linaria* Mill.
Sect. *Linaria*	§ *Grandes* Benth. p.p.max.	Sect. *Grandes* (Benth.) Wettst. p.p.max.	Sect. *Linaria* p.p.max.
	§ *Supinae* Benth. p.p.min.	Sect. *Supinae* (Benth.) Wettst. p.p.min.	
	§ *Diffusae* Benth. p.p.min.	Sect. *Diffusae* (Benth.) Wettst. p.p.min.	
Sect. *Speciosae* (Benth.) Wettst.	§ *Speciosae* Benth. p.p.max.	Sect. *Speciosae* (Benth.) Wettst. p.p.max.	Sect. *Speciosae* (Benth.) Wettst. p.p.
	§ *Versicolores* Benth. p.p.	Sect. *Versicolores* (Benth.) Wettst. p.p.	Sect. *Repentes* Valdés ex Viano
	§ *Diffusae* Benth. p.p.min.	Sect. *Diffusae* (Benth.) Wettst. p.p.min.	
Sect. *Diffusae* (Benth.) Wettst.	§ *Diffusae* Benth. p.p.	Sect. *Diffusae* (Benth.) Wettst. p.p.	Sect. *Diffusae* (Benth.) Wettst.
	§ *Minutiflorae* Benth.		Sect. *Minutiflorae* Benth. ex Kuprian.
	§ *Speciosae* Benth. p.p.	Sect. *Speciosae* (Benth.) Wettst. p.p.	Sect. *Speciosae* (Benth.) Wettst. p.p.
Sect. *Supinae* (Benth.) Wettst.:			
Subsect. *Supinae*	§ *Arvenses* Benth. p.p.max.	Sect. *Arvenses* (Benth.) Wettst. p.p.max.	Sect. *Arvenses* (Benth.) Wettst.
	§ *Supinae* Benth. p.p.	Sect. *Supinae* (Benth.) Wettst. p.p.	Sect. *Supinae* (Benth.) Wettst. subsect. *Supinae*
	§ *Diffusae* Benth. p.p.		Sect. *Supinae* subsect. *Amethystea* Valdés
	§ *Grandes* Benth. p.p.min.		Sect. *Linaria* p.p.min.
Subsect. *Saxatile* Valdés	§ *Supinae* Benth. p.p.	Sect. *Supinae* (Benth.) Wettst. p.p.	Sect. *Supinae* subsect. *Saxatile* Valdés
	§ *Versicolores* Benth. p.p.min.		Sect. *Bipunctatae* Viano p.p.max.
Subsect. *Trimerocalyx* (Murb.) D.A. Sutton	Not included	Not included	Not included
Sect. *Pelisserianae* Valdés	§ *Grandes* Benth. p.p.min.	Sect. *Grandes* (Benth.) Wettst. p.p.min.	Sect. *Pelisseriana* Valdés
	§ *Arvenses* Benth. p.p.min.	Sect. *Arvenses* (Benth.) Wettst. p.p.min.	
Sect. *Versicolores* (Benth.) Wettst.:			
Subsect. *Versicolores*	§ *Versicolores* Benth. p.p.	Sect. *Versicolores* (Benth.) Wettst. p.p.	Sect. *Versicolores* (Benth.) Wettst.
	§ *Diffusae* Benth. p.p.min.	Sect. *Diffusae* (Benth.) Wettst. p.p.	Sect. *Bipunctatae* Viano p.p.min.
	§ *Supinae* Benth. p.p.min.		
Subsect. *Elegantes* (Viano) D.A. Sutton	§ *Versicolores* Benth. p.p.min.	Sect. *Versicolores* (Benth.) Wettst. p.p.min.	Sect. *Elegantes* Viano
Sect. *Macrocentrum* D.A. Sutton	§ *Versicolores* Benth. p.p.min.	Sect. *Versicolores* (Benth.) Wettst. p.p.min.	Sect. *Versicolores* (Benth.) Wettst. p.p.min.
Nuttallanthus D.A. Sutton	§ *Versicolores* Benth. p.p.min.	Sect. *Versicolores* (Benth.) Wettst. p.p.min.	Sect. *Lectoplectron* Pennell

Note. p.p. = pro parte, p.p.max. = pro parte maxima, p.p.min. = pro parte minima.

Table 2

Major Features of Infrageneric Taxa of *Linaria* (according to Sutton 1988) and *Nuttallanthus*

	No. species	No. sampled species	Seed shape	Seed wing	Habit	Adaxial lobe of calyx	Stigma	Fruiting calyx	Palate development	Distribution
Linaria:										
Sect. *Linaria*	45	9	Discoid, laterally compressed	Wing	Perennial	Normal	Entire	5-partite	Prominent	Eurasia
Sect. *Speciosae*	19	14	Nondiscoid	No wing	Perennial	Normal	Entire	5-partite	Prominent	Mediterranean region
Sect. *Diffusae*	17	11	Nondiscoid	No wing	Annual or perennial	Normal	Entire	5-partite	Prominent	Mediterranean region
Sect. *Supinae*	44	38								
Subsect. *Supinae*	32	30	Discoid, laterally compressed	Wing or marginal ridge	Annual or perennial	Normal	Entire	5-partite	Prominent	Mediterranean region
Subsect. *Saxatile*	11	8	Nondiscoid	Wing, marginal ridge, or no wing	Annual or perennial	Normal	Entire	5-partite	Prominent	Mediterranean region
Subsect. *Trimerocalyx*	1	0	Discoid, laterally compressed	Wing	Annual	Normal	Entire	3-partite	Prominent	Northern Africa
Sect. *Pelisserianae*	2	2	Discoid, dorsiventrally compressed	Wing	Annual or perennial	Normal	Entire	5-partite	Prominent	Mediterranean region
Sect. *Versicolores*	21	19								
Subsect. *Versicolores*	19	17	Nondiscoid	No wing	Annual or perennial	Normal	Divided	5-partite	Prominent or weak	Mediterranean region
Subsect. *Elegantes*	2	2	Nondiscoid	No wing	Annual	Normal	Emarginate	5-partite	Weak	Iberian Peninsula
Sect. *Macrocentrum*	2	2	Nondiscoid	No wing	Annual	Reduced	Entire	5-partite	Weak	Mediterranean region
Nuttallanthus	4	3	Nondiscoid	No wing	Annual or biennial	Normal	Entire	5-partite	Weak	North and South America

test systematic and evolutionary hypotheses. Objectives were as follows: (1) to determine whether *Nuttallanthus* constitutes an evolutionary lineage distinct from *Linaria*; (2) to test the hypothesis of a basal divergence between two lineages of *Linaria*, one with winged seeds and the other with wingless seeds; and (3) to test the naturalness of the seven sections of Sutton's (1988) classification.

Material and Methods

Sampling Strategy and DNA Sequencing

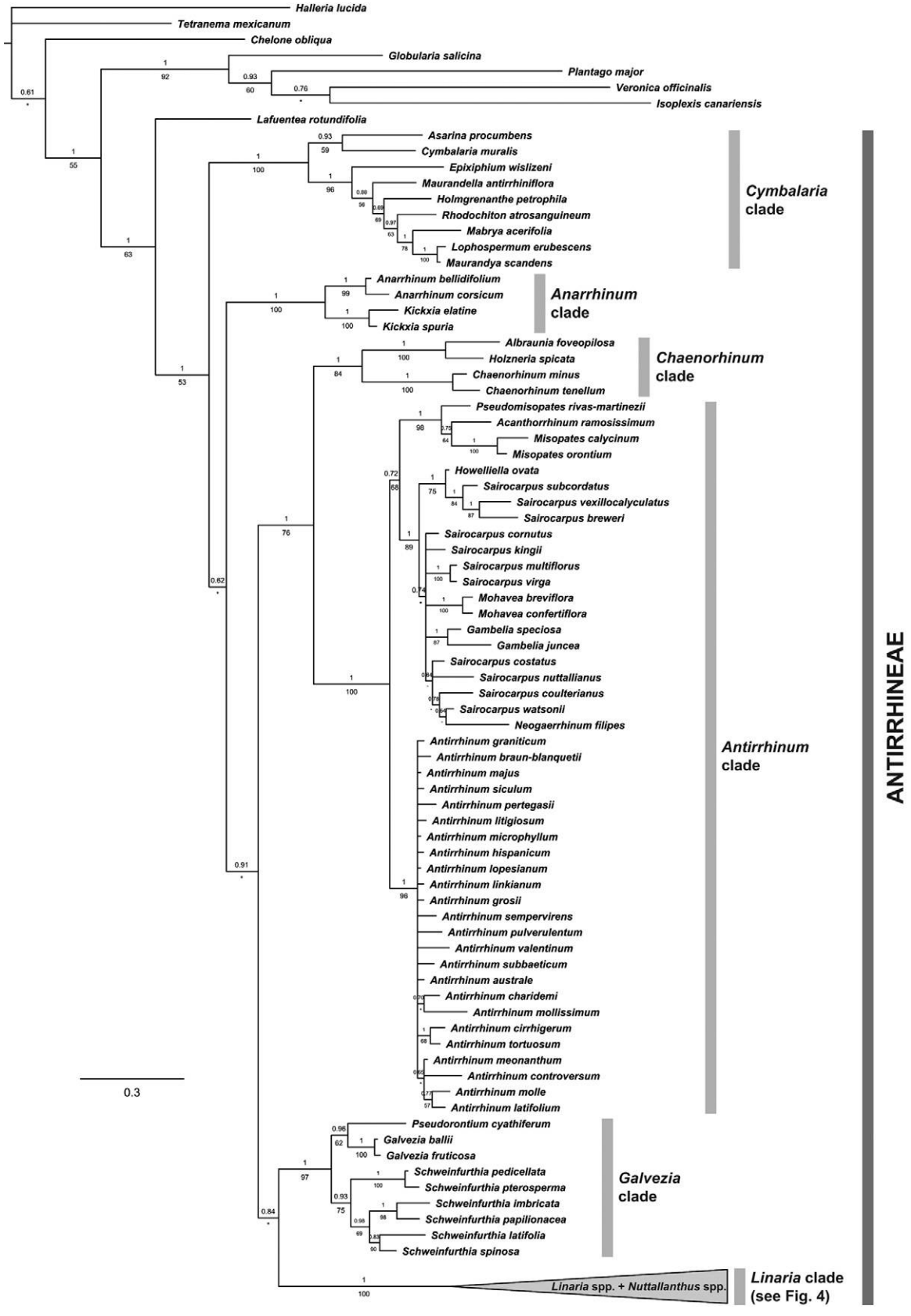
We sampled plants from 94 of ~150 species of *Linaria* and two of four species of *Nuttallanthus* (app. A). All sections of *Linaria* recognized in recent taxonomic accounts (Valdés 1970; Viano 1978*a*, 1978*b*; Sutton 1988) were represented. Plants were collected in the field and dried in silica gel or obtained from herbarium sheets (MA, RNG, E, UPOS, SEV, ATH). Total genomic DNA was extracted using the DNeasy Plant Mini kit (Qiagen, Valencia, CA). We amplified the ITS region (ITS1-5.8S-ITS2) of the nuclear ribosomal DNA using primer combinations 17SE-26SE (Sun et al. 1994) or ITS5-ITS4 (White et al. 1990; Sang et al. 1995). PCRs consisted of a 1-min pretreatment at 94°C and 30 cycles of 1 min at 94°C, 1 min at 50°–54°C, and 1 min at 72°C. Because of poor DNA quality obtained from herbarium specimens, in some cases the ITS1 and ITS2 regions were separately amplified using internal primers designed in the conserved 5.8S region: 5.8S-R (5′-GCGCAACTTGCGTTCAAAGA-3′) and 5.8S-F (5′-GCTCTCGCATCGATGAAGAA-3′). All amplified products were submitted to Macrogen (Seoul, South Korea) for sequencing using primers ITS5 and ITS4. Resulting sequence data were assembled and edited in Geneious Pro (ver. 5; Drummond et al. 2010) and submitted to GenBank (see app. A for accession numbers). Sequences of one additional species of *Nuttallanthus* (*N. canadensis*) and 71 species representing the remaining 26 genera of Antirrhineae recognized by Sutton (1988) and Vargas et al. (2004) were retrieved from the GenBank database (Oyama and Baum 2004; Vargas et al. 2004, 2009; C. E. Freeman, unpublished data), except for the sequence of *Maurandya scandens*, which was newly generated. We also retrieved from GenBank the ITS sequence of *Lafuentea rotundifolia*, which is closely related to Antirrhineae (Albach et al. 2005), and seven additional species representing as many Lamiales genera to be used as the outgroup following Vargas et al. (2004; app. A). The full data set was thus constituted by all 168 sampled sequences of Antirrhineae (including the 94 sequences of *Linaria*, three of *Nuttallanthus* and 71 of other genera) plus the eight additional sequences of Lamiales.

Phylogenetic Analyses

All sequences were aligned using MAFFT (ver. 6; Katoh et al. 2002) with default parameters, and further adjustments were made by visual inspection. Phylogenetic analyses were conducted using Bayesian inference (BI) and maximum likelihood (ML). *Halleria lucida* was used as the outgroup (as in Vargas et al. 2004). The best-fitting substitution model (GTR+G) was determined under the Akaike Information Criterion in jModelTest (ver. 0.1.1; Guindon and Gascuel 2003; Posada 2008). BI was performed in MrBayes (ver. 3.1.2; Ronquist and Huelsenbeck 2003) using two runs with four chains and 20 million generations each and a sample frequency of 1000. Stationarity and convergence of runs were confirmed with Tracer (ver. 1.4; Rambaut and Drummond 2007). A 50% majority-rule consensus tree with Bayesian posterior probabilities (PPs) of clades was calculated after removing the first 10% generations as burn in. ML was implemented in PhyML (ver. 3.0; Guindon and Gascuel 2003; Guindon et al. 2010) using the nearest neighbor interchange branch-swapping algorithm. One thousand nonparametric bootstrap replicates (BSs) were applied to assess clade supports. To evaluate the impact of different alignment methods on phylogenetic results, we realigned the matrix using the ClustalW (Larkin et al. 2007) plug in implemented in Geneious Pro (ver. 5), with no manual adjustments. The ClustalW alignment was then analyzed using the same phylogenetic methods (BI and ML).

Bayesian Hypothesis Testing

Bayes factors (BFs) allow testing of alternative hypotheses in a Bayesian framework (Kass and Raftery 1995; Suchard et al. 2001). They quantify the support for one hypothesis versus another given the data. We used this approach, as implemented in Tracer (ver. 1.4; Rambaut and Drummond 2007), to test alternative hypotheses about *Linaria* systematics and evolution. Tracer uses the harmonic mean estimator (HME) to compute Bayes factors (Newton and Raftery 1994; Kass and Raftery 1995). The HME has been claimed to be biased as it favors more parameter-rich models and has high variance (Lartillot and Philippe 2006). Nevertheless, it is widely used because of its computational tractability and easy implementation. Given that we compared models differing only in tree topology, we considered that the problems with the HME would not importantly bias our results. To reduce computing time, BF analyses were performed on a reduced data set including the 94 sequences of *Linaria* and three of *Nuttallanthus*, plus *Schweinfurthia latifolia* and *Galvezia fruticosa* as the outgroup (on the basis of the results of the full data set; see below). Six alternative phylogenetic and systematic hypotheses (constrained tree topologies in alternative MrBayes analyses) involving several taxonomical entities (Valdés 1970; Viano 1978*b*; Sutton 1988) were tested versus an unconstrained analysis H_0. Accordingly, monophyly constraints were set as follows: H_1, monophyly of genus *Linaria* (excluding American species); H_2, monophyly of sect. *Linaria*; H_3, monophyly of sect. *Diffusae*; H_4, monophyly of sect. *Speciosae*; H_5, monophyly of sect. *Supinae*; and H_6, monophyly of winged species and wingless species. Stationarity and convergence of analyses were assessed in Tracer after discarding the first 10% of sampled generations as burn in. Marginal likelihoods, their standard errors (estimated using 1000 bootstrap replicates), and BFs were obtained in Tracer. We considered 2xlnBF(H vs. H_0) −2 to −6 as positive evidence against H in favor of H_0, 2xlnBF(H vs. H_0) −6 to −10 as strong evidence against H in favor of H_0, and 2xlnBF(H vs. H_0) less than −10 as very strong evidence against H in favor of H_0 (Kass and Raftery 1995).



Results

Phylogenetic Analyses

After alignment with MAFFT and further manual adjustments, the data set had a total length of 677 bp with 397 variable and 300 parsimony-informative characters. The two runs of the Bayesian analysis yielded congruent topologies and similar posterior probabilities for supported clades (PP differences of <0.03). The consensus tree of the two runs is shown in figures 3 and 4. Overall, the ML analysis of the MAFFT alignment yielded a congruent topology, except for some weakly supported clades (figs. 3, 4). An overall congruent topology was also obtained in the phylogenetic analyses of the ClustalW alignment (see app. B, available in the online edition of the *International Journal of Plant Sciences*), except for the resolution of some basal clades of the Antirrhineae and the outgroup and some poorly supported clades within *Linaria*. There were, however, some differences in clade support (PP > 0.90): three clades that were statistically supported in the MAFFT analysis were not in the ClustalW analysis, and eight clades that were statistically supported in the ClustalW analysis were not in the MAFFT analysis. As manual adjustments were needed in the MAFFT alignment, conflicting statistical support for certain clades may have been the result of character homoplasy in the ClustalW analysis. Nevertheless, major phylogenetic patterns did not depend on the alignment method, as shown by the fact that all major clades of the Antirrhineae and *Linaria* were strongly supported by analyses of both alignments. For clarity, only results based on the MAFFT alignment and congruent with those of the ClustalW alignment are further discussed.

Monophyly of the Antirrhineae was strongly supported in the BI analysis (PP = 1; BS = 53%; fig. 3), with *Lafuentea* as sister taxon (PP = 1; BS = 63%). Six major well-supported clades were recognized within the Antirrhineae, which have been given the name of one representative genus: the *Cymbalaria* clade (9 genera; PP = 1; BS = 100%), the *Anarrhinum* clade (2 genera; PP = 1; BS = 100%), the *Chaenorhinum* clade (3 genera; PP = 1; BS = 84%), the *Antirrhinum* clade (9 genera; PP = 1; BS = 100%), the *Galvezia* clade (3 genera; PP = 1; BS = 97%), and the *Linaria* clade, constituted by all sampled species of *Linaria* and *Nuttallanthus* (PP = 1; BS = 100%). Relationships among these clades were weakly supported except for the sister-group relationship between the *Chaenorhinum* and *Antirrhinum* clades (PP = 1; BS = 76%).

Within the *Linaria* clade, six major clades (A–F) were recognized by the two phylogenetic analyses. Clades A, B, and C were respectively formed by the two species of sect. *Macrocentrum* (PP = 1; BS = 100%), the two species of sect. *Pelisserianae* (PP = 1; BS = 96%), and the three sampled species of *Nuttallanthus* (PP = 1; BS = 100%). All 19 sampled species of sect. *Versicolores* constituted clade D (PP = 1; BS = 99%). Clade E (PP = 1; BS = 90%) was formed by all sampled species of sect. *Linaria*, all sampled species of sect. *Speciosae*, and four species of sect. *Diffusae*. The latter four taxa formed a well-supported monophyletic lineage (PP = 1; BS = 100%). Finally, clade F (PP = 1; BS = 95%) was formed by the remaining species of sect. *Diffusae* and all sampled species of sect. *Supinae*.

Relationships between major clades were poorly resolved except for the sister-group relationship between clades E and F (PP = 1; BS = 97%). A sister-group relationship between clades B and C was supported mainly by BI (PP = 1; BS = 66%). On the other hand, relationships between clades A, B + C, D, and E + F remained unsupported in the two analyses.

Bayesian Hypothesis Testing

The Bayes factor analyses (table 3) recovered decisive (very strong) support (2xlnBF less than −10) for rejection of four out of six phylogenetic hypotheses: H_3 (monophyly of sect. *Diffusae*), H_4 (monophyly of sect. *Speciosae*), H_5 (monophyly of sect. *Supinae*), and H_6 (monophyly of winged species and wingless species). Hypotheses H_1 (monophyly of genus *Linaria*) and H_2 (monophyly of sect. *Linaria*) were not decisively but positively (2xlnBF −2 to −6) and strongly (2xlnBF −6 to −10) rejected, respectively.

Discussion

Our deep sampling of *Linaria* and *Nuttallanthus*, as well as the addition of five genera (*Mabrya*, *Maurandya*, *Holmgrenanthe*, *Epixiphium*, and *Galvezia*) to previously analyzed ITS sequences (Oyama and Baum 2004; Vargas et al. 2004, 2009) provided the most deeply sampled phylogenetic hypothesis of the Antirrhineae tribe published to date (figs. 3, 4). Phylogenetic relationships among Antirrhineae genera herein reported are congruent with those obtained by Vargas et al. (2004) based on the same DNA region. Therefore, we are for the first time able to place all Antirrhineae genera within the six major clades of Antirrhineae previously recognized (fig. 3; Vargas et al. 2004) and to assess phylogenetic relationships of *Linaria* and *Nuttallanthus* in a wide evolutionary framework.

Monophyly of Toadflaxes, Including New World Species

Phylogenetic naturalness of *Linaria* was first suggested by Vargas et al. (2004) on the basis of a strongly supported monophyletic group of only eight sampled species representing the seven sections of Sutton's classification. This analysis did not, however, include any sample of the American toadflaxes (genus *Nuttallanthus*). In our ITS phylogeny (figs. 3, 4), all sampled species of the two toadflax genera (*Linaria* and *Nuttallanthus*)

Fig. 3 Phylogenetic analysis of internal transcribed spacer sequences of Antirrhineae (168 sequences, including 94 of *Linaria* and three of *Nuttallanthus*). The 50% majority-rule consensus tree obtained in the Bayesian analysis of the MAFFT alignment is shown. Numbers above branches are Bayesian posterior probabilities. Numbers below branches are maximum likelihood percentage bootstrap values. An asterisk indicates no bootstrap support over 50% but presence of the clade in the maximum likelihood tree. A hyphen indicates no bootstrap support over 50% and absence of the clade in the maximum likelihood tree. Phylogenetic relationships within the *Linaria* clade (collapsed here) are shown in fig. 4.

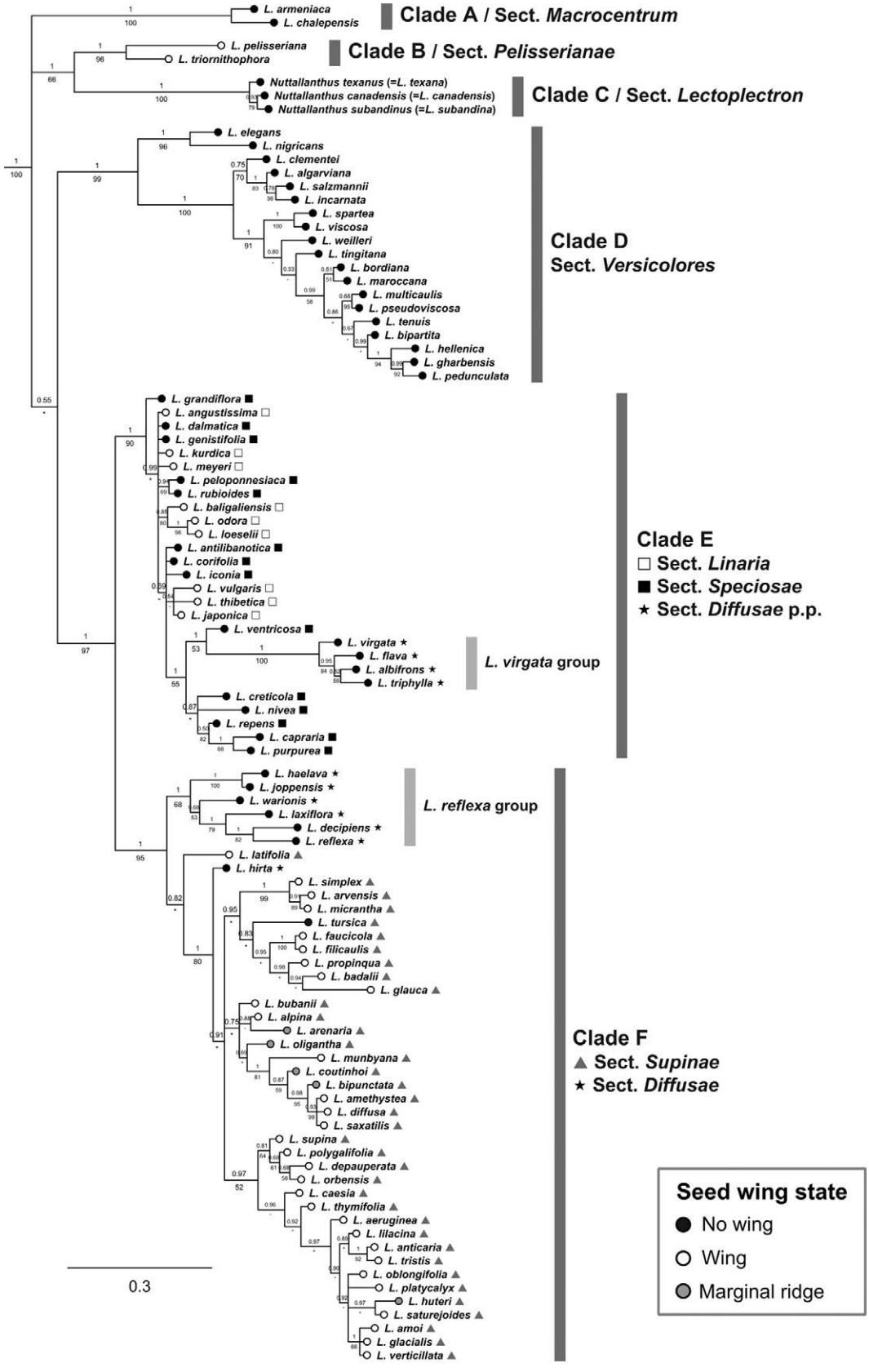


Table 3

Mean Values of Marginal Likelihood and Bayes Factor Test Statistics (2xlnBF) for the Unconstrained Analysis (H_0) and the Tested Phylogenetic Hypotheses (H_1–H_8)

Hypothesis	Marginal likelihood (lnP(model | data)) ± SE	2xlnBF(H vs. H_0)
H_0	−5523.562 ± .307	...
H_1: monophyly of genus *Linaria*	−5525.144 ± .340	−3.164
H_2: monophyly of sect. *Linaria*	−5526.706 ± .343	−6.288
H_3: monophyly of sect. *Diffusae*	−5570.774 ± .381	−94.424
H_4: monophyly of sect. *Speciosae*	−5556.528 ± .325	−65.932
H_5: monophyly of sect. *Supinae*	−5530.160 ± .342	−13.196
H_6: monophyly of winged species and wingless species	−5761.062 ± .323	−475.000

Note. 2xlnBF(H vs. H_0) −2 to −6 reflects positive evidence against H in favor of H_0, 2xlnBF(H vs. H_0) −6 to −10 reflects strong evidence against H in favor of H_0, and 2xlnBF(H vs. H_0) less than −10 reflects very strong evidence against H in favor of H_0 (Kass and Raftery 1995).

formed a strongly supported monophyletic group. Monophyly of toadflaxes is also supported by the basic chromosome number x=6 and a set of morphological traits that are not found together elsewhere in the Antirrhineae: presence of hypocotylary stems; entire, sessile, pinnately veined leaves; terminal, bracteate, racemose inflorescences; and spurred flowers (Valdés 1970; Sutton 1988).

North American toadflaxes were classically included within *Linaria* (Chavannes 1833; Bentham 1846; Wettstein 1895; Diels 1906; Pennell 1935). However, Sutton (1988) argued that separation of the four American species as a distinct genus was justified on the basis of several morphological traits of flowers and seeds: the prismatic seeds with four to seven ridges, the abaxial lip of the corolla greatly exceeding the adaxial lip, the weakly developed palate that barely occludes the corolla tube, and a very slender or absent spur. However, seed morphology is highly variable across *Linaria* sections (Sutton 1988), and flower morphology of *Nuttallanthus* species is noticeably similar to that found in the two species of *Linaria* sect. *Macrocentrum* and some species of sect. *Versicolores* (see the poorly developed palates and slender spurs of *L. nigricans*, *L. armeniaca*, and *N. texanus* in fig. 1*V*, 1*Y*, and 1*Z*, respectively). In our phylogenetic analyses (fig. 4), the genus *Linaria* was recovered as a paraphyletic group, with the three sampled species of *Nuttallanthus* (including the type species *N. canadensis*) nested within it (clade C; fig. 4). A sister-group relationship between *Nuttallanthus* and *Linaria* sect. *Pelisserianae* was strongly supported by the Bayesian analysis and was moderately supported by the ML analysis. In addition, Bayes factors (table 3) provided positive evidence against the monophyly of *Linaria* excluding *Nuttallanthus*. Therefore, on the basis of morphological traits and phylogenetic results (fig. 4; table 3), it would probably be appropriate to circumscribe *Nuttallanthus* species as a section of *Linaria*. This approach was first proposed by Pennell (1919, 1935), who treated the group as sect. *Lectoplectron*, treatment later followed by Valdés (1970). Nevertheless, given the poor resolution at the base of the *Linaria* clade, the moderate support of the *Nuttallanthus-Linaria* sect. *Pelisserianae* clade in the ML analysis and the fact that the present analysis was based on a single DNA region, additional data are desirable to definitely reject a sister-group relationship and reciprocal monophyly of *Linaria* and *Nuttallanthus*.

Support for the Infrageneric Classification of Linaria

Viano's (1978*a*, 1978*b*) hypothesis of *Linaria* evolution—that is, a basal dichotomy in which species with wingless and winged seeds constitute two natural sister lineages—was clearly rejected by our results (fig. 4; table 3). Conversely, several shifts in seed morphology (between wingless seeds, winged seeds, and seeds with a marginal ridge; fig. 2) appear to have occurred in the course of *Linaria* evolution (see fig. 4), as also indicated by the nonhomologous anatomy of winged seeds across the genus (Sutton 1988). When comparing the main lineages of our phylogeny with recent infrageneric classifications of *Linaria* (fig. 4; tables 1, 2), it is apparent that some sections that are well defined by distinct morphological traits were also found to be monophyletic. This is the case of sects. *Macrocentrum*, *Pelisserianae*, and *Versicolores*. Conversely, the remaining sections (*Supinae*, *Linaria*, *Speciosae*, *Diffusae*) were not supported as monophyletic groups in our analyses.

Sect. *Macrocentrum*. The two species of sect. *Macrocentrum* (*L. chalepensis* and *L. armeniaca*, clade A; fig. 1*X*, 1*Y*) display some unusual traits that are unique or rare within *Linaria* and that led Sutton (1980) to separate them from sect. *Versicolores* (where they had been included before; Bentham 1846). First of all, in *L. chalepensis* and *L. armeniaca* the adaxial lobe of the calyx is shorter than the remaining four abaxial lobes. This trait is not found elsewhere in *Linaria* but also occurs in *Holzneria*, which is a genus not closely related to *Linaria* according to nuclear (fig. 3; see also Vargas et al. 2004) and plastid (P. Vargas et al., unpublished results) phylogenies. Second, the small, lateral appendage present at

Fig. 4 Phylogenetic relationships of 94 sampled species of *Linaria* and three of *Nuttallanthus*, on the basis of the analysis of internal transcribed spacer sequences. The *Linaria* clade obtained in the 50% majority-rule consensus tree of the Bayesian analysis of Antirrhineae sequences is shown (see fig. 3 for the rest of the Antirrhineae tree). Numbers above branches are Bayesian posterior probabilities. Numbers below branches are maximum likelihood percentage bootstrap values. An asterisk indicates no bootstrap support over 50% but presence of the clade in the maximum likelihood tree. A hyphen indicates no bootstrap support over 50% and absence of the clade in the maximum likelihood tree. Delimitation of sections follows Sutton (1988), except for sect. *Lectoplectron*, which follows Valdés (1970).

the base of each stamen filament is considered unique within the Antirrhineae (Sutton 1980). And third, seeds have five or six longitudinal angles, a trait shared with *Nuttallanthus* but not with the other sections of *Linaria*.

Sect. *Pelisserianae*. This section is constituted by two sister species (*L. triornithophora* and *L. pelisseriana*, clade B; fig. 1*Q*, 1*R*), which are rather different in terms of habit, as well as disposition, size, and shape of flowers and leaves. *Linaria triornithophora* and *L. pelisseriana* had been respectively placed in sects. *Grandes* (=sect. *Linaria*) and *Arvenses* (Bentham 1846; Wettstein 1895) until Valdés (1970) reunited them on the basis of morphology and structure of capsules and seeds. While the discoid seeds of other winged-seeded species of *Linaria* are laterally compressed, in *L. triornithophora* and *L. pelisseriana* seeds are dorsiventrally compressed (Sutton 1988). This peculiar pattern of seed development is not found elsewhere in the genus and appears to be a synapomorphy of sect. *Pelisserianae*. Despite the apparent sister-group relationship of sects. *Pelisserianae* and *Lectoplectron* (=*Nuttallanthus*), there are no evident morphological similarities connecting them. Indeed, extensive morphological changes may have occurred in both lineages since their common ancestor, given their likely old divergence (as suggested by the long branches separating them).

Sect. *Versicolores*. The morphological distinctness of sect. *Versicolores*, as defined by Sutton (1988; clade D; fig. 1*S*–1*W*), was considered to be based on a diagnostic morphological synapomorphy not found elsewhere in the genus or even the tribe: a divided style with discrete stigmatic areas. A bifid style is clearly observed in the majority of species, while a merely emarginated stigma is found in *L. elegans* and *L. nigricans*. Viano (1978*a*, 1978*b*) recognized these two groups as independent sections on the basis of stigma and seed morphology, while Sutton (1988) included both of them in sect. *Versicolores*. The two groups were revealed as monophyletic and sister to each other in our ITS phylogeny, which is in agreement with a previous cpDNA phylogeny (Fernández-Mazuecos and Vargas 2011). Naturalness of sect. *Versicolores* is further supported by a particular pattern of seedling development (Champagnat 1961).

Sects. *Diffusae* (*L. virgata* Group), *Linaria*, and *Speciosae*. Unlike sects. *Macrocentrum*, *Pelisserianae*, *Versicolores*, and *Lectoplectron* (=*Nuttallanthus*), the remaining four sections of *Linaria* were not resolved as monophyletic in our phylogenetic analyses. Relationships among species of sects. *Linaria* (fig. 1*A*–1*C*) and *Speciosae* (fig. 1*D*–1*F*) were poorly resolved in clade E. Additionally, the BF tests yielded strong and very strong evidence against the monophyly of sects. *Linaria* and *Speciosae*, respectively (table 3). Despite notable differences in seed shape (winged in sect. *Linaria* and wingless in sect. *Speciosae*), Sutton (1988) already indicated a close morphological relationship between species of both sections on the basis of the perennial habit, erect stems, and similar leaf, flower, and capsule morphology. Four species of sect. *Diffusae* were also included in clade E (*L. virgata*, *L. albifrons*, *L. flava*, and *L. triphylla*; henceforth, "the *L. virgata* group"; figs. 1*G* and 1*H*). The inclusion of the *L. virgata* group in the same clade as sects. *Linaria* and *Speciosae* is interesting from a systematic standpoint. The species of sect. *Diffusae* have wingless seeds, an unreduced adaxial lobe of calyx, and an undivided style. These traits are shared with sect. *Speciosae*. In fact, sects. *Diffusae* and *Speciosae* cannot be clearly differentiated, although the first is mainly formed by annuals that usually have procumbent or ascending fertile stems, while the second is formed by perennials that usually have erect fertile stems. The naturalness of sect. *Diffusae* was questioned by both Valdés (1970) and Sutton (1988). Valdés (1970) was the first to suggest that this section was probably polyphyletic, while Sutton (1988) considered that it was formed by a heterogeneous group of species that would have affected the delimitation of other sections. Moreover, Sutton recognized the *L. virgata* group as a distinctive morphological complex within *Diffusae*, which is congruent with the distinct phylogenetic position of this group in our phylogeny. Unlike most other species of sect. *Diffusae*, species of the *L. virgata* group display very prominent anticlinal walls to the testa cells and a sunken periclinal wall with no papilla (Sutton 1988).

Since we obtained low phylogenetic resolution within clade E and we analyzed a low number of species of sect. *Linaria* (nine of 45), additional markers and further taxon sampling are needed to reveal the phylogenetic relationships within this lineage.

Sects. *Diffusae* (*L. reflexa* Group) and *Supinae*. Six of the remaining species of sect. *Diffusae*, including the type species *L. reflexa* (henceforth, "the *L. reflexa* group"; fig. 1*I*), formed a monophyletic group within clade F. Clade F also included two basal species (*L. latifolia*, sect. *Supinae*, fig. 1*K*; and *L. hirta*, sect. *Diffusae*, fig. 1*J*), followed by a clade of the other 36 species of sect. *Supinae*. *Linaria latifolia* is differentiated from the remaining species of sect. *Supinae* by several morphological features, such as erect stems, large flowers, broad leaves, and long and slender calyx lobes. These singular traits of *L. latifolia* resemble those of sect. *Linaria* (stems and flowers) and sect. *Pelisserianae* (calyx lobes). Nevertheless, *L. latifolia* has been usually confused with *L. hirta* (sect. *Diffusae*) because of their similar stems, leaves, and flowers, whereas seed shape and inflorescence indumentum are the main features to tell apart the two species.

Monophyly of the remaining species of sect. *Supinae* (excluding *L. latifolia*; fig. 1*L*–1*P*) was obtained in the two phylogenetic analyses, although with statistical support only in the BI analysis. Nevertheless, the naturalness of sect. *Supinae* was strongly supported by a recently published study (Blanco-Pastor et al. 2012) in which the combination of both nuclear and plastid sequences in a coalescent-based analysis allowed the detection of hybridization and incomplete lineage sorting. These two processes seem to have obscured the phylogenetic signal within this group as obtained from individual gene trees.

No evident morphological synapomorphies were found for clade F. Although the presence of a fertile primary (epicotylary) stem together with heteromorphic—fertile and sterile—secondary (hypocotylary) stems connects sects. *Diffusae* and *Supinae* (Sutton 1988), these traits are also found in *Diffusae* species of the unrelated *L. virgata* group (clade E). In the remaining sections, either the primary stem soon degenerates (sects. *Linaria*, *Macrocentrum*, *Pelisserianae*, and *Versicolores*) or stems are homomorphic (sects. *Speciosae* and *Linaria*).

Conclusions

Our ITS phylogeny provided the first phylogenetic insights into the evolution and systematics of toadflaxes. Monophyly of toadflaxes including Palearctic (*Linaria*) and American (*Nuttallanthus*) species was supported. The *Linaria* clade therefore constitutes a fourth New World–Old World lineage of Antirrhineae (see Vargas et al. 2004). A basal divergence between species with winged and wingless seeds was clearly unsupported, which implies the homoplasy of this trait. Congruence between distinctive morphological characters and well-supported ITS lineages suggested that sects. *Macrocentrum*, *Pelisserianae*, and *Versicolores* constitute distinct evolutionary lineages and should be maintained in systematics of *Linaria*. The American genus *Nuttallanthus* is also a natural group within the *Linaria* clade. Future studies will determine the appropriateness of including *Nuttallanthus* in *Linaria* as a section (sect. *Lectoplectron*). In contrast, our results cast doubt on the naturalness of sects. *Supinae*, *Linaria*, *Speciosae*, and *Diffusae* as recognized by Sutton (1988; but see Blanco-Pastor et al. 2012 for sect. *Supinae*). Polyphyly of sect. *Diffusae* had already been suggested on the basis of morphology. Our phylogeny agrees with the recognition of at least two natural groups: the *L. reflexa* group, which would remain as sect. *Diffusae* (with *L. reflexa* as the type species), and the *L. virgata* group, which could be treated as sect. *Minutiflorae*, a taxon recognized by some authors and typified by *L. albifrons* (Bentham 1846; Valdés 1970). In any case, further molecular markers and analyses would be needed before firmly establishing a new sectional classification of all *Linaria* species.

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Appendix A

Voucher Information and GenBank Accession Numbers of ITS Sequences

The following information is provided for each sampled species of *Linaria*, *Nuttallanthus*, and the outgroup: taxon; distribution; sampled locality; voucher; and GenBank accession number of ITS sequence.

Acanthorrhinum ramosissimum (Cosson & Durieu) Rothm.; N Africa; Morocco, road from Ouarzazate to Zagora; VAL 41469; AY731261. ***Albraunia foveopilosa*** Speta; SW Iran; Iran, Khuzistan, Baghmalek-Haftgel; TARI 38909; AY731250. ***Anarrhinum corsicum*** Jordan & Fourr.; Corsica; France, Corsica; Podlech 47340 (A); AF513881. ***A. bellidifolium*** (L.) Willd.; W Europe; Natural Botanical Garden of Dublin; VAL 145150; AY731263. ***Antirrhinum australe*** Rothm.; S Spain; Spain, Granada, Castril; VAL 140895; AY731273. ***A. braun-blanquetii*** Rothm.; N Iberian Peninsula; Spain, Palencia, Cervera de Pisuerga; VAL 35121; AY731269. ***A. charidemi*** Lange; SE Spain; Spain, Almería, Cabo de Gata; VAL 37158; AY731282. ***A. cirrhigerum*** Welw. ex Ficalho; W Mediterranean region; Morocco, Doukkala-Abda, El Jadida; VAL 111299; EU677200. ***A. controversum*** Pau; SE Spain; Spain, Albacete, Villa de Ves; VAL 145152; AY731272. ***A. graniticum*** Rothm.; C Spain-E Portugal; Spain, Madrid, Fuentidueña del Tajo; VAL 99540; AY731283. ***A. grosii*** Font Quer; CW Spain; Spain, Ávila, Sierra de Gredos; VAL 37049; AY731281. ***A. hispanicum*** Chav.; S Spain; Spain, Granada, Veleta road; P. Vargas 120PV99; AY731286. ***A. latifolium*** Miller; NE Spain to C Italy; Spain, Lérida, Bapà; VAL 144658; AY731274. ***A. linkianum*** Boiss.; Portugal; Portugal, Sintra; VAL 144655; AY731278. ***A. litigiosum*** Pau; SE Spain; Spain, Valencia, Serra; VAL 144656; AY731271. ***A. lopesianum*** Rothm.; NE Portugal; Portugal, Vimioso, Carçao; F. Amich & S. Bernardo s.n.; EU677217. ***A. majus*** L.; SW Europe; Spain, Lérida, Valle de Arán; VAL 144657; AY731280. ***A. meonanthum*** Hoffmanns. & Link; N Portugal; Spain, Ávila, El Tremedal; P. Vargas 149PV99; AY731284. ***A. microphyllum*** Rothm.; EC Spain; Spain, Guadalajara, Entrepeñas; VAL 40051; AY731267. ***A. molle*** L.; NE Spain; Spain, Huesca, Sopeira; VAL 35176; AY731268. ***A. mollissimum*** Rothm.; SE Spain; Spain, Almería, Sierra de Gádor; VAL 37143; AY731275. ***A. pertegasii*** Rothm.; E Spain; Spain, Castellón, Cova Fosca; J. Güemes JG4092; EU677226. ***A. pulverulentum*** Lázaro; E Spain; Spain, Zaragoza, Nuévalos; VAL 31592; AY731279. ***A. sempervirens*** Lapeyr.; Pyrenees; Spain, Huesca, Panticosa; VAL 145148; AY731270. ***A. siculum*** Miller; CE Mediterranean region; Italy, Sicily, Messina; VAL 119899; AY731276. ***A. subbaeticum*** Güemes, Mateu & Sánchez Gómez; SE Spain; Spain, Albacete, Bogarra, El Batán; J. Güemes JG4081; AY731287. ***A. tortuosum*** Boscex Vent.; CE Mediterranean region; Italy, Ancona, Sirolo; VAL 39871; AY731285. ***A. valentinum*** Font Quer; E Spain; Spain, Valencia, La Safor; VAL 39799; AY731266. ***Asarina procumbens*** Miller; NE Spain-S France; Botanischer Garten Berlin-Dahlem; VAL 145146; AF513879. ***Chaenorhinum minus*** (L.) Lange; Europe-SW Asia; unknown; McNeils 96–336 (GH); AF513875. ***C. tenellum*** (Cav.) Lange; E Spain; Spain, Valencia, Moixent; VAL 37839; AY731251. ***Chelone obliqua*** Mix; N America; unknown; Wolfe 586 (OS); AF375164. ***Cymbalaria muralis*** P. Gaertner et al.; S Europe; Switzerland; Nyffeler R. s.n.; AF513883. ***Epixiphium wislizeni*** (A. Gray) Munz; SW North America; USA,

Texas, El Paso Co.; UTEP 56828; AY878930. ***Galvezia ballii*** Munz; NW Perú; Perú, Piura; Cowan 4487 (TEX); AY492104. ***G. fruticosa*** J.F. Gmelin; Perú; unknown; Dillon 3776 (GH); AF513885. ***Gambelia juncea*** (Bentham) D.A. Sutton; SW North America; unknown; C.E. Freeman s.n.; AY316310. ***G. speciosa*** Nutt.; SW North America; unknown; C.E. Freeman s.n.; AY316310. ***Globularia salicina*** Lam.; Canary Islands; cultivated; Chase 2547 (K); AF313039. ***Halleria lucida*** L.; S África; unknown; Wolfe 684 (OS); AF375149. ***Holmgrenanthe petrophila*** (Coville & C.V. Morton) Elisens; SW USA; USA, California, Inyo Co.; UTEP 67327; AY880231. ***Holzneria spicata*** (Korovin) Speta; SW Asia; Iran, Khorasan, Tobart-e Sefid; TARI 23577; AY731258. ***Howelliella ovata*** (Eastw.) Rothm.; SW North America; USA, Califórnia; Thompson 434 (GH); AF513899. ***Isoplexis canariensis*** (L.) Loud.; Canary Islands; cultivated; Chase s.n. (K); AF313033. ***Kickxia elatine*** (L.) Dumort.; Eurasia and N Africa; Spain, Barcelona, Sant Pere de Ribes-Sitges; VAL 41793; AY731265. ***K. spuria*** (L.) Dumort.; Eurasia and N Africa; Spain, Valencia, Chera; VAL 37098; AY731264. ***Lafuentea rotundifolia*** Lag.; S Iberian Peninsula; Spain; Martínez Ortega 889 (SALA); AF509816. ***Linaria aeruginea*** (Gouan) Cav.; Iberian Peninsula; Spain, Granada; J.L. Blanco-Pastor 51JB09 (MA); JQ814486. ***L. albifrons*** (Sibth. & Sm.) Steudel; SW Asia, S and E Mediterranean; Israel, Negev A. Danin, S.G. Knees et al. (RNG); JX481129. ***L. algarviana*** Chav.; SW Portugal; Portugal, Cabo de São Vicente; M. Fernández-Mazuecos 11MF09 (MA); JX481086. ***L. alpina*** (L.) Mill.; CS Europe; Spain, Huesca; S. Martín Bravo 571SMB05 (UPOS); JQ814489. ***L. amethystea*** (Vent.) Hoffmanns. & Link; SW Europe, NW Africa; Spain, Ciudad Real; R. García Río (MA 712742); JQ814490. ***L. amoi*** Campo ex Amo; S Spain; Spain, Málaga; J.L. Blanco-Pastor 37JB09 (MA); JX481108. ***L. angustissima*** (Loisel.) Borbás; S and C Europe; Romania, Cluj-Napoca; J. Güemes and G. Bacchetta (MA 618431); JX481133. ***L. anticaria*** Boiss. & Reut.; S Spain; Spain, Málaga; J.L. Blanco-Pastor 33JB09 (MA); JQ814491. ***L. antilibanotica*** Rech.f.; SW Asia; Lebanon, Baalbek; K. Sleem (RNG); JX481134. ***L. arenaria*** DC.; W France; France, Vendée; F. De Raeve (RNG); JX481112. ***L. armeniaca*** Chav.; SW Asia; Armenia, Gegharkunik; C. Aedo et al. (MA 743447); JX481080. ***L. arvensis*** (L.) Desf.; SWC Europe, NW Africa, SW Asia; Spain, Almería; S.L. Jury and R.N. Carter (RNG); JQ814494. ***L. badalii*** Loscos; NW Spain; Spain, Leon; M.F. Gardner and S.G. Gardner (RNG); JQ814495. ***L. baligaliensis*** Patzak; Afghanistan; Afghanistan, Salong Pass; J.F. Veldkamp (MA 784820); JX481142. ***L. bipartita*** (Vent.) Willd.; W Morocco; Morocco, Rabat; S.L. Jury and R.G. Wilson 18558 (RNG); JX481094. ***L. bipunctata*** (L.) Chaz.; NC Portugal; Spain, Soria; A. Segura (RNG); JQ814496. ***L. bordiana*** Santa & Simonneau; NW Africa; Algeria, Sidi Lakhdar; D.A. and S.J. Sutton 172 (RNG); JX481095. ***L. bubanii*** Font Quer; NE Spain; Spain, Huesca; M. Carrasco (MA 609430). JQ814537/JQ814538. ***L. caesia*** (Pers.) F.G. Dietr.; C Spain; Spain, Ciudad Real; A. Molina and J. Varela (RNG); JX481105. ***L. capraria*** Moris & De Not.; Italy; Italy, Marciana; R. M. Baldini and L. Vivona (MA 693545); JX481146. ***L. chalepensis*** (L.) Mill.; NC and E Mediterranean; Cyprus, Cape Kiti; Iter Mediterraneum IV (MA 495681); JX481081. ***L. clementei*** Haens.; S Spain; Spain, Málaga, Alhaurín de la Torre; M. Fernández-Mazuecos et al. 7MF08 (MA); JX481089. ***L. corifolia*** Desf.; SW Asia; Turkey, Dogançal; J.J. Aldasoro (MA 689911); JX481135; ***L. coutinhoi*** Valdés; N Portugal; Portugal, Freixo-de-Espada; A. Teixeira s.n. (MA); JX481113. ***L. creticola*** Kuprian.; Rússia; Russia, Belgorod; V. Gladkova and T. Leonova (E 00419502); JX481143. ***L. dalmatica*** (L.) Mill.; SC Europe, SW Asia; Bulgaria, Rhodopes Mountains; C. Navarro et al. (MA 726987); JX481136. ***L. decipiens*** Batt.; Algeria; Algeria, l'Akfadou NP; A. Dubois (MA 589738); JX481125. ***L. depauperata*** Leresche ex Lange; SE Spain; Spain, Albacete; P.F. Cannon et al. (RNG); JX481107. ***L. diffusa*** Hoffmanns. & Link; NC Portugal; Portugal, Freixo-de-Espada; A. Teixeira s.n. (MA); JX481114. ***L. elegans*** Cav.; W Iberia; Portugal, Manteigas; M. Fernández-Mazuecos 127MF10 (MA); JX481103. ***L. faucicola*** Leresche & Levier; NW Spain; Spain, León; F. Llamas et al. (MA 619920); JX481117. ***L. filicaulis*** Boiss. ex Leresche & Levier; CN Spain; Spain, León; C.M. Romero Rodríguez (MA 789283); JX481118. ***L. flava*** (Poiret) Desf.; Algeria, Corsica, Sardinia; Italy, Corsica; C. Bukanell and L. Ollum (E 00419551); JX481130. ***L. genistifolia*** (L.) Mill.; CE Europe, W Asia; Turkey, Hadim-Bezkir; J.J. Aldasoro (A9751); JX481137. ***L. gharbensis*** Batt. & Pit.; NW Africa, SW Spain; Spain, Huelva, Gibraleón; Fernández-Mazuecos et al. 7MF09 (MA); JX481100. ***L. glacialis*** Boiss.; S Spain; Spain, Granada; J.L. Blanco-Pastor 43JB09 (MA); JQ814504. ***L. glauca*** (L.) Chaz.; CE Spain; Spain, Madrid J. Calvo (MA 790863); JX481116. ***L. grandiflora*** Desf.; SE Europe, SW Asia; Turkey, Erzurum; A. Herrero et al. (MA 687558); JX481151. ***L. haelava*** (Forskål) F.G. Dietr.; NE Africa, SW Asia; Israel, Horbat Medin; D. Heller and I. Shammash (MA 532177); JX481122. ***L. hellenica*** Turrill; Greece; Greece, Kambos; unknown collector (ATH); JX481102. ***L. hirta*** (L.) Moench; S Iberian Peninsula; Spain, Zamora; P. Bariego (MA 793918); JX481120. ***L. huteri*** Lange; S Spain; Spain, Málaga; J.L. Blanco-Pastor 32JB09 (MA); JX481111. ***L. iconia*** Boiss. & Heldr.; Turkey; Turkey, Konya; Gordon C. Hillman (RNG); JX481154. ***L. incarnata*** (Vent.) Spreng.; SW Iberia, NW Africa; Spain, Salamanca, Pelabravo; M. Fernández-Mazuecos and P. Vargas 39MF09 (MA); JX481088. ***L. japonica*** Miq.; E Asia; Japan, Tottori; S. and T. Taniguchi s.n. (MA); JX481141. ***L. joppensis*** Bornm.; Israel; Israel, Ashkeleton; A. Danin, S.G. Knees et al. (RNG); JX481123. ***L. kurdica*** Boiss. & Hohen.; SW Asia; Armenia, Vayots Dzor; A. Herrero et al. (MA 744412); JX481139. ***L. latifolia*** Desf.; SW Spain, NW Africa; Spain, Sevilla; Ladero and Rivas Goday (SEV 32442); JX481124. ***L. laxiflora*** Desf.; N Africa; Tunisia, Jerid, Cedada; C. Aedo et al. (MA 795183); JX481128. ***L. lilacina*** Lange; SE Spain; Spain, Jaén; J.L. Blanco-Pastor 16JB12 (MA); JX481156. ***L. loeselii*** Schweigger; NW Europe; Lithuania, Apskritis ol Klaipeda; E. Glazkova and A. Quintanar (MA 791644); JX481153. ***L. maroccana*** Hook.f.; Morocco; Morocco, Marrakech–Tizi-n-Test; S.L. Jury et al. 14209 (RNG); JX481097. ***L. meyeri*** Kuprian.; Russia; Georgia, Mtskhete Mtianeti; L. Muñoz et al. (MA 764400); JX481150. ***L. micrantha*** (Cav.) Hoffmanns. & Link; Mediterranean, SW Asia; Spain, Huelva; J.L. Blanco-Pastor 22JB09 (MA); JQ814513. ***L. multicaulis*** (L.) Mill.; N Africa, Sicilia; Italy, Sicily, Etna; I. Álvarez et al. IA1622 (MA); JX481098. ***L. munbyana*** Boiss. & Reut.; W Mediterranean; Spain, Huelva; J.L. Blanco-Pastor 21JB09 (MA); JQ814515. ***L. nigricans*** Lange; SE Spain; Spain, Almería, Tabernas; P. Vargas 3PV08 (MA); JX481104. ***L. nivea*** Boiss. & Reut.; Spain; Spain, Toledo; C. Aedo (MA 611701); JX481155. ***L. oblongifolia*** (Boiss.) Boiss. & Reut.; S Iberian Peninsula; Spain, Málaga; J.L. Blanco-Pastor 34JB09 (MA); JQ814516. ***L. odora*** (Bieb.)

Fisch.; Russia; Russia, Voilgograd; A.K. Skvortsov (MA 618431); JX481152. ***L. oligantha*** Lange; SE Spain; Spain, Alicante; L. Serra (MA 753096); JX481121. ***L. orbensis*** Carretero & Boira; E Iberian Peninsula; Spain, Alicante, Sagra; J.L. Blanco-Pastor 4JB10; JQ814518. ***L. pedunculata*** (L.) Chaz.; W Mediterranean; Spain, Huelva, Marismas del Odiel; M. Fernández-Mazuecos et al. 4MF09 (MA); JX481101. ***L. pelisseriana*** (L.) Mill.; CE Mediterranean, W Europe; Turkey, Bayramiç; S. Castroviejo (MA 643850); JX481082. ***L. peloponnesiaca*** Boiss. & Heldr.; Balkans; Greece, Mount Olympus; P. Vargas (MA 778352); JX481148. ***L. platycalyx*** Boiss.; Spain; Spain, Cádiz; S. Martín Bravo 5SMB08 (UPOS); JQ814520. ***L. polygalifolia*** Hoffmanns. & Link; Iberian Peninsula; Portugal, Monte Gordo; J.L. Blanco-Pastor 33JB10 (MA); JQ814522. ***L. propinqua*** Boiss. & Reut.; N Spain; Spain, Bilbao; J.A. Alejandre (MA 468162); JQ814524. ***L. pseudoviscosa*** Murb.; Tunisia; Tunisia, El Haouaria; P. Wilkin and E.J. Wellens 231 (RNG); JX481099. ***L. purpurea*** (L.) Mill.; Italy; United Kingdom, Norwich (cultivated); M. Fernández-Mazuecos (74MF09); JX481147. ***L. reflexa*** (L.) Chaz.; N Africa; Algeria, Algiers; J.J. Aldasoro A9799 (MA); JX481126. ***L. repens*** (L.) Mill.; W Europe; Spain, Cuenca, El Tobar; M. Fernández-Mazuecos (54MF09); JX481144. ***L. rubioides*** Vis. & Pančić; Balkans; Serbia, Mokra Gora; S.L. Jury (RNG); JX481149. ***L. salzmannii*** Boiss.; S Spain; Spain, Málaga, El Chorro; M. Fernández-Mazuecos and J. Ramírez 19MF09 (MA); JX481087. ***L. saturejoides*** Boiss.; S Iberian Peninsula; Spain, Málaga; J.L. Blanco-Pastor 36JB09 (MA); JQ814525. ***L. saxatilis*** (L.) Chaz.; NC Iberian Peninsula; Spain, Madrid; P. Vargas 20PV09 (MA); JX481115. ***L. simplex*** Willd. ex Desf.; S Europe, N Africa, SW Asia; Greece, Ararchova; P. Vargas 79PV08 (MA); JQ814528. ***L. spartea*** (L.) Chaz.; SW Europe; Spain, Madrid, Colmenar; P. Vargas 101PV07 (MA); JX481090. ***L. supina*** (L.) Chaz.; SW Europe; France, Gorges de l'Hérault; J. Lambinon (RNG 2009/12/131); JQ814530. ***L. tenuis*** (Viv.) Spreng.; NC and NE Africa; Libya, Tripoli; Davis and Boulos 50581 (RNG); JX481096. ***L. thibetica*** Franch.; China; China, Sichuan; D.E. Boufford et al. (E 00292244); JX481140. ***L. thymifolia*** (Vahl) DC.; SW France; France, Gironde; B. de Retz (MA 303566); JX481106. ***L. tingitana*** Boiss. & Reut.; NW Africa; Algeria, El Macta; D.A. and S.J. Sutton 383 (RNG); JX481092. ***L. triornithophora*** (L.) Willd.; W Iberian Peninsula; Spain, Cáceres, Sierra de Gata; M. Fernández-Mazuecos (18MF07); JX481083. ***L. triphylla*** (L.) Mill.; W and S Mediterranean; Tunisia, El Vef; J. Calvo et al. (MA 797461); JX481132. ***L. tristis*** (L.) Mill.; S Spain, NW Africa; Spain, Cádiz; P. Jiménez Mejías 105PJM04 (UPOS); JX481109. ***L. tursica*** Valdés & Cabezudo; SW Spain; Spain, Huelva; J.L. Blanco-Pastor 18JB09 (MA); JQ814533. ***L. ventricosa*** Coss. & Bal.; Morocco; Morocco, Errachidia; T. Buira, J. Calvo and S. Hantson (MA 807960); JX481145. ***L. verticillata*** Boiss.; S Spain; Spain, Granada; J.M. Losa (RNG 2009/12/53); JX481110. ***L. virgata*** (Poiret) Desf.; N Africa; Algeria, SE Constantine; D.A and S.J. Sutton (RNG); JX481131. ***L. viscosa*** (L.) Chaz.; S and W Iberia; Spain, Huelva, Marismas del Odiel; M. Fernández-Mazuecos et al. 6MF09 (MA); JX481091. ***L. vulgaris*** Mill.; Eurasia; France, Chamonix; B. Estébanez s.n.; JX481138. ***L. warionis*** Pomel; NW Africa; Morocco, Beni Tajjita; D. Podlech (MA 589733); JX481127. ***L. weilleri*** Emb. & Maire; S Morocco; Morocco, Tirhmi; Miller, Russell and Sutton s.n. (RNG); JX481093. ***Lophospermum erubescens*** D. Don; México; Botanicher Garten Berlin-Dahlem; VAL 145154; AY731249. ***Mabrya acerifolia*** (Pennell) Elisens; SW USA; USA, Arizona, Maricopa Co.; UTEP 56309; AY878934. ***Maurandella antirrhiniflora*** (Willd.) Rothm.; E Mexico; Mexico; Hill 18323 (GH); AF513878. ***Maurandya scandens*** (Cav.) Pers.; C America; cultivated; P. Vargas 1103; JX481079. ***Misopates calycinum*** (Vent.) Rothm.; SW Iberian Peninsula and N Africa; Spain, Canary Islands, Lanzarote; ORT s/n; AY731259. ***M. orontium*** (L.) Raf.; Mediterranean region; Spain, Valencia, Serra; VAL 145155; AY731260. ***Mohavea breviflora*** Coville; SW USA; unknown; Hileman L. s.n.; AF513892. ***M. confertiflora*** (A. DC.) A.A. Heller; SW North America; USA, California; Hileman L. s.n.; AF513891. ***Neogaerrhinum filipes*** (A. Gray) Rothm.; SW North America; USA, California; Thompson 254 (GH); AF513896. ***Nuttallanthus canadensis*** (L.) D.A.Sutton [=*Linaria canadensis* (L.) Dum.Cours.]; USA, Canada; USA, Alabama, Pike County; C.E. Freeman (UTEP 65885); AY883085. ***N. subandinus*** (Diels) D.A.Sutton [=*Linaria subandina* Diels]; S America; Brazil, São Francisco de Paula; Grazziotin and Perazzolo (MA 406417); JX481084. ***N. texanus*** (Scheele) D.A.Sutton [=*Linaria texana* Scheele]; USA, Mexico; USA, Del Mar Mesa; D.E. Breedlove (MA 494665); JX481085. ***Plantago major*** L.; Eurasia; New Zealand; WELTU 20180; FJ024619. ***Pseudomisopates rivas-martinezii*** (Sánchez Mata) Güemes; CW Spain; Spain, Ávila, Sierra de Gredos, Conventos creek; P. Vargas 377–99; AY731262. ***Pseudorontium cyathiferum*** (Bentham) Rothm.; SW North America; USA, California; Van Devender 92–268 (AZ); AF513893. ***Rhodochiton atrosanguineum*** (Zucc.) Rothm.; C Mexico; Bergius Botanical Garden; VAL 145153; AF513876. ***Sairocarpus breweri*** (A. Gray) D.A. Sutton; SW USA; USA, California, Tehama Co.; UTEP 66786; AY880229. ***S. cornutus*** (Bentham) D.A. Sutton; SW USA; unknown; Oyama RK 12 (A); AF513905. ***S. costatus*** (Wiggins) D.A. Sutton; NW Mexico; unknown; VanDevender 92-268 (AZ); AF513893. ***S. coulterianus*** (A. DC.) D.A. Sutton; SW North America; unknown; #16273 (RSA); AF513890. ***S. kingii*** (S. Watson) D.A. Sutton; SW USA; unknown; Morefield 3382 (GH); AF513903. ***S. multiflorus*** (Pennell) D.A. Sutton; SW USA; unknown; Oyama RK 5 (A); AF513897. ***S. nuttallianus*** D.A. Sutton; SW North America; USA, California; Oyama RK 27 (A); AF513895. ***S. subcordatus*** (A. Gray) D.A. Sutton; SW USA; unknown; Oyama RK 79 (A); AF513902. ***S. vexillocalyculatus*** (Kellog) D.A. Sutton; SW USA; unknown; Oyama RK 73 (A); AF513900. ***S. virga*** (A. Gray) D.A. Sutton; SW USA; unknown; Oyama RK 6 (A); AF513898. ***S. watsonii*** (Vasey & Rose) D.A. Sutton; NW Mexico; unknown; Fishbein 3136 (AZ); AF513894. ***Schweinfurthia imbricata*** A.G. Miller et al.; E Oman; Oman, Wadi Bed; E 99215; AY731254. ***S. latifolia***; Baker ex Oliver; Yemen; Yemen, Hadramout, Wadi 'Aidid; E 99214; AY731255. ***S. papilionacea*** (L.) Boiss.; SW Asia; Oman, Near Muscat; E 46435; AY731253. ***S. pedicellata*** (T. Anderson) Balf. fil.; NE Africa-SW Asia; Socotra, Ras Bashorah; E 99213; AY731256. ***S. pterosperma*** (A. Rich.) A. Braun; NE Africa and SW Asia; unknown; Thulin 8205 (UPS); AF513882. ***S. spinosa*** A.G. Miller et al.; Oman; Oman, Dhofar, Manston to Mudhai; E 99203; AY731257. ***Tetranema mexicanum*** Benth.; C America; unknown; Wolfe s.n. (OS); AF375151. ***Veronica officinalis***; Eurasia; UK, Farthing Downs; Chase s.n. (K); AF313012.

Literature Cited

Albach DC, HM Meudt, B Oxelman 2005 Piecing together the "new" Plantaginaceae. Am J Bot 92:297–315.

Beninger CW, RR Cloutier, B Grodzinski 2009 A comparison of antirrhinoside distribution in the organs of two related Plantaginaceae species with different reproductive strategies. J Chem Ecol 35:1363–1372.

Bentham G 1846 Scrophulariaceae. Pages 186–598 *in* A De Candolle, ed. Prodromus systematis naturalis regni vegetabilis. Vol 10. Sumptibus Victoris Masson, Paris.

Blanco-Pastor JL, P Vargas, BE Pfeil 2012 Coalescent simulations reveal hybridization and incomplete lineage sorting in Mediterranean *Linaria*. PLoS ONE 7:e39089.

Boissier E 1879 Flora orientalis. Vol 4. AH Georg, Geneva.

Box MS, S Dodsworth, PJ Rudall, RM Bateman, BJ Glover 2011 Characterization of *Linaria* KNOX genes suggests a role in petal-spur development. Plant J 68:703–714.

Champagnat M 1961 Recherches de morphologie descriptive et expérimentale sur le genre *Linaria*. Ann Sci Nat Bot Biol Veg 12: 1–170.

Chavannes É 1833 Monographie des antirrhinées. C Treuttle et Würtz, Paris.

Cubas P, C Vincent, E Coen 1999 An epigenetic mutation responsible for natural variation in floral symmetry. Nature 401: 157–161.

Desfontaines R 1798 Flora atlantica. Vol 2. LG Desgranges, Paris.

Diels FE 1906 Plantae novae andinae imprimis Weberbauerianae I. 15. Scrophulariaceae andinae (Calceolariis exceptis). Bot Jahrb Syst 37:427–433.

Drummond AJ, B Ashton, M Cheung, J Heled, M Kearse, R Moir, S Stones-Havas, S Sturrock, T Thierer, A Wilson 2010 Geneious version 5.0. http://www.geneious.com/.

Dumortier BC 1827 Florula belgica, operis majoris prodromus. Tornaci Nerviorum, Staminacia.

Fernández-Mazuecos M, P Vargas 2011 Historical isolation versus recent long-distance connections between Europe and Africa in bifid toadflaxes (*Linaria* sect. *Versicolores*). PLoS ONE 6:e22234.

Galego L, J Almeida 2007 Molecular genetic basis of flower colour variegation in *Linaria*. Genet Res 89:129–134.

Ghebrehiwet M, B Bremer, M Thulin 2000 Phylogeny of the tribe Antirrhineae (Scrophulariaceae) based on morphological and *ndh*F sequence data. Plant Syst Evol 220:223–239.

Guindon S, JF Dufayard, V Lefort, M Anisimova, W Hordijk, O Gascuel 2010 New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol 59:307–321.

Guindon S, O Gascuel 2003 A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 52:696–704.

Kass RE, AE Raftery 1995 Bayes factors. J Am Stat Assoc 90:773–795.

Katoh K, K Misawa, K Kuma, T Miyata 2002 MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res 30:3059–3066.

Lamarck JB, AP De Candolle 1805 Flore française. Vol 1. CH Agasse, Paris.

Lange J 1870 Scrophulariaceae. Pages 546–620 *in* M Willkomm, J Lange, eds. Prodromus florae Hispanicae. Vol 2. SE Schweizerbart (E Koch), Stuttgart.

Larkin MA, G Blackshields, NP Brown, R Chenna, PA McGettigan, H McWilliam, F Valentin, et al 2007 Clustal W and Clustal X version 2.0. Bioinformatics 23:2947–2948.

Lartillot N, H Philippe 2006 Computing Bayes factors using thermodynamic integration. Syst Biol 55:195–207.

Linnaeus C 1753 Species plantarum. Vol 2. Impensis Laurentii Salvii, Stockholm.

Miller P 1754 The gardeners dictionary. 4th ed. Vol 2. J Rivington and J Rivington, London.

Morison R 1680 Plantarum historiae universalis Oxoniensis. Vol 2. E Theatro Sheldoniano, Oxford.

Newton MA, AE Raftery 1994 Approximate Bayesian inference with the weighted likelihood bootstrap. J Royal Statistical Soc B (Methodological) 56:3–48.

Oyama RK, DA Baum 2004 Phylogenetic relationships of North American *Antirrhinum* (Veronicaceae). Am J Bot 91: 918–925.

Pennell FW 1919 Scrophulariaceae of the Local Flora. II. Torreya 19: 143–152.

——— 1935 The Scrophulariaceae of eastern temperate North America. Acad Nat Sci Phila Monogr 1:1–650.

Posada D 2008 jModelTest: phylogenetic model averaging. Mol Biol Evol 25:1253–1256.

Rambaut A, AJ Drummond 2007 Tracer version 1.4. http://beast.bio.ed.ac.uk/Tracer.

Ronquist F, JP Huelsenbeck 2003 MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574.

Rothmaler W 1943 Zur Gliederung der Antirrhineae. Feddes Repert 52:16–39.

Sáez L, M Bernal 2009 *Linaria* Mill. Pages 232–324 *in* S Castroviejo, A Herrero, C Benedí, E Rico, J Güemes, eds. Flora iberica. Vol 13. CSIC, Madrid.

Sánchez-Lafuente AM, MA Rodríguez-Gironés, R Parra 2011 Interaction frequency and per-interaction effects as predictors of total effects in plant-pollinator mutualisms: a case study with the self-incompatible herb *Linaria lilacina*. Oecologia 168:153–165.

Sang T, DJ Crawford, TF Stuessy 1995 Documentation of reticulate evolution in peonies (*Paeonia*) using internal transcribed spacer sequences of nuclear ribosomal DNA: implications for biogeography and concerted evolution. Proc Natl Acad Sci USA 92:6813–6817.

Schwarz-Sommer Z, B Davies, A Hudson 2003 An everlasting pioneer: the story of *Antirrhinum* research. Nat Rev Genet 4:655–664.

Sing SE, RKD Peterson 2011 Assessing environmental risks for established invasive weeds: dalmatian (*Linaria dalmatica*) and yellow (*L. vulgaris*) toadflax in North America. Int J Environ Res Public Health 8:2828–2853.

Suchard MA, RE Weiss, JS Sinsheimer 2001 Bayesian selection of continuous-time Markov chain evolutionary models. Mol Biol Evol 18:1001–1013.

Sun Y, DZ Skinner, GH Liang, SH Hulbert 1994 Phylogenetic analysis of *Sorghum* and related taxa using internal transcribed spacers of nuclear ribosomal DNA. Theor Appl Genet 89:26–32.

Sutton DA 1980 A new section of *Linaria* (Scrophulariaceae: Antirrhineae). Bot J Linn Soc 81:169–184.

——— 1988 A revision of the tribe Antirrhineae. Oxford University Press, Oxford.

Tournefort JP 1700 Institutiones rei herbariae. E Typographia Regia, Paris.

Valdés B 1970 Revisión de las especies europeas de *Linaria* con semillas aladas. An Univ Hipalense 7:1–288.

Vargas P, E Carrió, B Guzmán, E Amat, J Güemes 2009 A geographical pattern of *Antirrhinum* (Scrophulariaceae) speciation

since the Pliocene based on plastid and nuclear DNA polymorphisms. J Biogeogr 36:1297–1312.

Vargas P, JA Rosselló, R Oyama, J Güemes 2004 Molecular evidence for naturalness of genera in the tribe Antirrhineae (Scrophulariaceae) and three independent evolutionary lineages from the New World and the Old. Plant Syst Evol 249:151–172.

Viano J 1978*a* Les linaires à graines aptères du bassin méditerranéen occidental. 1. *Linaria* sect. *Versicolores*. Candollea 33:33–88.

——— 1978*b* Les linaires à graines aptères du bassin méditerranéen occidental. 2. *Linaria* sect. *Elegantes*, *Bipunctatae*, *Diffusae*, *Speciosae*, *Repentes*. Candollea 33:209–267.

Wettstein R 1895 Scrophulariaceae. Pages 39–107 *in* A Engler, K Prantl, eds. Die natürligen pflanzenfamilien. Vol 4. Lepzig.

White TJ, T Bruns, S Lee, J Taylor 1990 Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315–322 *in* M Innis, D Gelfand, J Sninsky, T White, eds. PCR protocols: a guide to methods and applications. Academic Press, San Diego, CA.

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Appendix B from Fernández-Mazuecos et al., "A Phylogeny of Toadflaxes (*Linaria* Mill.) Based on Nuclear Internal Transcribed Spacer Sequences: Systematic and Evolutionary Consequences"

(Int. J. Plant Sci., vol. 174, no. 2, p. 234)

Supplemental Figure

FIG. B1.—Phylogenetic analysis of ClustalW-aligned internal transcribed spacer sequences of Antirrhineae (168 sequences, including 94

of Linaria and three of Nuttallanthus). The 50% majority-rule consensus tree obtained in the Bayesian analysis is shown. Numbers above branches are Bayesian posterior probabilities. Numbers below branches are maximum likelihood percentage bootstrap values. An asterisk indicates no bootstrap support over 50% but presence of the clade in the maximum likelihood tree. A hyphen indicates no bootstrap support over 50% and absence of the clade in the maximum likelihood tree.

Manuscript 2

Blanco-Pastor *et al.* 2012, "Coalescent Simulations Reveal Hybridization and Incomplete Lineage Sorting in Mediterranean *Linaria*"

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Coalescent Simulations Reveal Hybridization and Incomplete Lineage Sorting in Mediterranean *Linaria*

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Abstract

We examined the phylogenetic history of *Linaria* with special emphasis on the Mediterranean sect. *Supinae* (44 species). We revealed extensive highly supported incongruence among two nuclear (ITS, AGT1) and two plastid regions (*rpl32-trnL*^{UAG}, *trnS-trnG*). Coalescent simulations, a hybrid detection test and species tree inference in *BEAST revealed that incomplete lineage sorting and hybridization may both be responsible for the incongruent pattern observed. Additionally, we present a multilabelled *BEAST species tree as an alternative approach that allows the possibility of observing multiple placements in the species tree for the same taxa. That permitted the incorporation of processes such as hybridization within the tree while not violating the assumptions of the *BEAST model. This methodology is presented as a functional tool to disclose the evolutionary history of species complexes that have experienced both hybridization and incomplete lineage sorting. The drastic climatic events that have occurred in the Mediterranean since the late Miocene, including the Quaternary-type climatic oscillations, may have made both processes highly recurrent in the Mediterranean flora.

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Introduction

Gene trees can differ from one another and do not always correspond to species trees [1–4]. Wendel and Doyle [5] listed three categories of processes that may cause incongruent patterns: technical causes, organism-level processes and gene- or genome-level processes. If technical causes, selection, paralogy and recombination can be ruled out, then (i) hybridization among fully differentiated species with subsequent fixation of nuclear and/or organellar loci and (ii) the incomplete random sorting of alleles at many loci independently due to short intervals between divergence events (hereafter incomplete lineage sorting) often remain as the main hypotheses that can explain gene tree incongruence [6–11]. Typically, phylogenetic analyses using single locus datasets (e.g. [12–14]) or concatenated datasets (e.g. [15–18]) have provided inferences of relationships in numerous plant groups. Nonetheless, a tree based on a single locus or concatenated genes may lead to a spurious representation of the history of the species [19,20]. Several methods that distinguish hybridization from incomplete lineage sorting have been recently described [21–23]. However, many independent loci are needed for their implementation and hybridization is difficult to uncover if multiple reticulation events have occurred. Ané *et al.* [24] implemented a method that can accommodate any source of incongruence even using a limited number of loci, but this method is unable to determine the process causing incongruence among phylogenies. Also, Maureira-Butler *et al.* [6] and Joly *et al.* [10] have proposed statistical frameworks, applicable to datasets with few independent loci, where hybridization can be detected in the presence of incomplete lineage sorting. Alternatively, several models can estimate the correct species tree if incongruence is due to incomplete lineage sorting alone [20,25–29], but in such models hybridization signals need to be previously ruled out or excluded. If not, an incorrect species tree may be inferred by such methods [20,30].

Both polyploid and homoploid hybrid speciation might represent a large fraction of the source of plant biodiversity on Earth [31]. In the Mediterranean basin, several plant groups suffered secondary contacts in their postglacial colonization routes from their glacial maximum refugia located in southern peninsulas [32] or after altitudinal migrations in restricted areas within peninsulas (e.g. Iberian Peninsula, [33,34]). A considerable proportion of the present Mediterranean plant diversity may be the result of hybridization episodes, which *per se* represent a challenge for phylogenetic reconstruction. Besides this, species complexes that underwent rapid speciation also represent a major challenge for molecular systematics. In those groups species relationships could be obscured by the ancestral polymorphisms retained through speciation events as a consequence of incomplete lineage sorting [2,35]. In the Mediterranean region, rapid plant speciation has been recently detected [36–38] and associated with adaptation to the establishment of the Mediterranean climatic rhythm (summer drought) (3.2 Ma) or the Quaternary-type Mediterranean climatic fluctuations (2.3 Ma) [39].

Toadflaxes (*Linaria* Mill.) constitute the largest genus within the snapdragon lineage (tribe Antirrhineae). *Linaria* comprises c.150

species that are widely distributed in the Palearctic region, but the genus is most diverse in the Mediterranean basin. The origin of the genus has been placed in the Miocene [40] predating the Messinian Salinity Crisis [41]. The monophyly of *Linaria* has been suggested based on nrDNA (ITS) sequences of eight species representing all sections [42], however, whether the sections constitute natural groups remains uncertain. Numerous taxonomic treatments of *Linaria* have been proposed [43–51], but remarkable disagreement in the infrageneric classification suggests complex evolutionary processes. The latest classification of the genus recognizes seven sections (*Linaria*, *Speciosae*, *Diffusae*, *Supinae*, *Pelisserianae*, *Versicolores* and *Macrocentrum*) [45]. Section *Supinae* (Benth.) Wetts. (hereafter *Supinae*) is a clear example of the systematic complexity within *Linaria* because of the disagreement in taxonomic treatments (Table 1). *Supinae* comprises 44 diploid (2n = 12) [52] hermaphroditic annual and perennial species differentiated from other sections by their laterally-compressed winged seeds that have a horizontal arrangement in globose capsules [45]. *Supinae* species are distributed in the temperate regions of Europe, northern Africa and western Asia (circum-Mediterranean distribution), with the highest diversity found in the Iberian Peninsula (40 species) [44,45].

In *Linaria*, hybrid species have been historically described when intermediate characters of two species meet in a plant [53,54]. In section *Supinae* several natural hybrids have been previously reported [44,55–57]. Artificial experiments have also shown the potential of hybridization inasmuch as *Supinae* species that do not meet in nature can produce capsules after hand cross-pollination ((Blanco-Pastor, unpublished), [53]). The highest fertilization success was found in crosses among *Supinae* species (13 successful crosses of 20 assayed), followed by clearly lower values in inter-sectional crosses (four successful crosses of 14) [53]. A lack of internal reproductive barriers among *Supinae* species is then suggested. Despite this, external barriers such as allopatry do exist at the present time within *Supinae* as few species have overlapping distributions. However, such geographical barriers may have not existed during glaciations.

The high chance for hybridization in *Linaria* may affect phylogenetic reconstruction in this genus. Nonetheless, incomplete lineage sorting cannot be discarded as a cause of phylogenetic incongruence. Both processes can be difficult to distinguish, but may also occur simultaneously [58]. Within this framework, we investigate causes of incongruence between three presumably unlinked loci. Two nuclear (ITS and AGT1) and two linked plastid (*rpl32-trnL*^{UAG} and *trnS-trnG*) regions are herein sequenced for *Linaria*, with special emphasis in *Supinae* species. Our aims are: (i) to test for the presence of reticulation signals by simulations under the coalescent model using the method of Maureira-Butler *et al.* [6], (ii) to detect individuals that may have been affected by historical hybridization (hereafter potential hybrids), (iii) to exclude potential hybrids and infer the species tree using a method that accounts for incomplete lineage sorting (*BEAST) [20], (iv) to compare the *BEAST species tree with our original gene trees to identify random sorting episodes, and (v) to recover the reticulation events by locating the parental lineages of the potential hybrids in a multilabelled species tree. The ultimate goal is to disclose the evolutionary history of *Supinae* by exploring the presence of incomplete lineage sorting and/or reticulation events that may have occurred during the course of the evolution of this plant group.

Materials and Methods

Sampling Strategy

Individuals were collected in the field and dried in silica gel or obtained from herbaria (MA, E, RNG) (Table S1). Total genomic DNA was extracted using the Dneasy Plant Mini Kit (QUIAGEN Inc., California). We amplified (using an Eppendorf Mastercycler Epgradient S, Westbury, NY) a low copy nuclear gene intron (AGT1) [59], the nuclear ribosomal internal transcribed spacer (ITS) [60] and two plastid regions (*rpl32-trnL*^{UAG}, *trnS-trnG*) [61,62] in 52 individuals representing 46 *Linaria* species plus one individual of *Antirrhinum* and one individual of *Chaenorhinum*. In particular, we used one species of sect. *Macrocentrum* (*L. chalepensis*), three species of sect. *Versicolores* (*L. spartea*, *L. gharbensis*, *L. multicaulis*), five species of sect. *Linaria* (*L. meyeri*, *L. loeselii*, *L. odora*, *L. thibetica*, *L. vulgaris*), four species of sect. *Speciosae* (*L. ventricosa*, *L. dalmatica*, *L. peloponnesiaca*, *L. genistifolia*), seven species of sect. *Diffusae* (*L. albifrons*, *L. flava*, *L. triphylla*, *L. laxiflora*, *L. warionis*, *L. haelava*, *L. joppensis*) and 24 of the 44 species of section *Supinae* [45]. We followed Sutton's species delimitation [45] for the non-Iberian species and Sáez & Bernal's delimitation [44] for the Iberian species but with minor changes

Table 1. Systematic classification of *Linaria* sect *Supinae* suggested in this study and its relation with previous classifications.

Bentham (1846)	Wettstein (1895)	Valdés (1970) and Viano (1978)	Sutton (1988), Sáez (2008)	Present study
Linaria sect. *Linariastrum* Chav.	*Linaria* Juss.	*Linaria* Miller	Sect. *Supinae* (Bentham) Wettst.	Sect. *Supinae* (Bentham) Wettst.
§ *Arvenses* Bentham p.p.max.	Sect. *Arvenses* (Bentham) Wettst. p.p.max.	Sect. *Arvenses* (Bentham) Wettst.	Subsect. *Supinae* p.p.	Subsect. *Arvenses*
§ *Supinae* Bentham p.p. § *Diffusae* Bentham p.p. § *Grandes* Bentham p.p.min.	Sect. *Supinae* (Bentham) Wettst. p.p.	Sect. *Supinae* (Bentham) Wettst. subsect. *Supinae*	Subsect. *Supinae* p.p.	Subsect. *Supinae*
§ *Supinae* Bentham p.p. § *Versicolores* Bentham p.p.min.	Sect. *Supinae* (Bentham) Wettst. p.p.	Sect. *Supinae* subsect. *Saxatile* Valdés p.p. max Sect. *Supinae* (Bentham) Wettst. subsect. *Supinae* p.p. Sect. *Supinae* subsect. *Amethystea* Valdés Sect. *Bipunctatae* Viano p.p.max.	Subsect. *Saxatile* Valdés Subsect. *Supinae* p.p.	Subsect. *Saxatile* Valdés
–	–	–	Subsect. *Trimerocalyx* (Murb.) – D.A. Sutton	

p.p. = *pro parte*.
p.p.max = *pro parte maxima*.
p.p.min = *pro parte minima*.
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regarding the "*Linaria verticillata* group" and the "*Linaria alpina* group" [44,63] (see Methods S1). We also included one additional species neither considered by Sutton nor Sáez & Bernal: *L. almijarensis* Campo & Amo [64] (see Table S1). All necessary permits were obtained for the described field studies. In cases where plant locations were protected we obtained permissions from the "Consejería de Medio Ambiente" of Andalusian Government (Spain), references: GB-86/2010/EA/FL/FA/JMLV, ENSN/JSG/IHC/MCF. Amplification products were outsourced for sequencing to a contract sequencing facility (Macrogen, Seoul, South Korea) on an ABI Prism® 3730xi DNA sequencer, using the same primer set as for PCR. Sequence data were edited using Geneious software (Biomatters Ltd., Auckland, New Zealand). Sequences are available in GenBank (see Table S1).

Deciphering of Haplotypes in Unphased Genotypes

More than one allele was found in both AGT1 and ITS in Sanger sequenced PCR amplicons. To decipher these, we first estimated the gametic phases of the sequences using Arlequin 3.5.1.2 [65]. This program performs a Gibbs sampling via the ELB algorithm [66] to obtain the posterior probability of phased haplotypes. The settings for the ELB algorithm were as follows: dirichlet alpha value: 0.01, epsilon value: 0.1, heterozygote site influence zone: 5, gamma value: 0.01, sampling interval: 500, no. of samples: 2000, burn-in steps: 100000 and 0% of recombination steps. AGT1 haplotypes retrieved with posterior probability under 0.95 were confirmed by cloning the purified PCR products using the Promega Corporation protocol (Madison, USA) with JM109 High Efficiency competent cells and pLysS plasmids. Four single recombinant colonies from each reaction were screened. Amplifications were performed using the T7-SP6 plasmid primers. All ITS haplotypes inferred with Arlequin were used to build allele trees. In only one case (*L. bubanii)* ITS haplotypes were not inferred as sister (or very closely related) sequences in the gene trees. As the phase posterior probability for this individual was low (0.41), we empirically confirmed the *L. bubanii* ITS haplotypes by sequencing the PCR product using allele-specific primers as described in Scheen *et al.* [67].

Test for Recombination

Recombination was tested within ITS and AGT1 datasets using RDP 3.44 [68] with the following methods: RDP [69], Geneconv [70], MaxChi [71], Bootscan/Recscan [72], SisScan [73], 3Seq [74] and Chimaera [75]. We selected 0.05 as the p-value cut-off in general settings and internal references only in the RDP method. A window size of 150 and step size of 20 was used in the Bootscan and SisScan methods and a variable window size was set in MaxChi and Chimaera methods. We considered that recombination was likely if it was accepted by more than two methods. For the remaining settings we used the default values.

Gene Trees Estimation and Calculation of Dates

The haplotype sequences obtained from the three datasets (ITS, AGT1, cpDNA) were analyzed by Bayesian Inference in MrBayes 3.1.2 [76] after alignment with MAFFT v.6 [77] (with corrections by visual inspection) and optimal substitution model selection in jModeltest 0.1.1 [78,79].

For time calibration, we used the divergence time between *Antirrhinum* and *Linaria* (13.33–27.32 Ma) from a previous estimate obtained in a relaxed molecular-clock analysis of tribe Antirrhineae (Vargas *et al.*, unpublished). This analysis was in turn calibrated with five Lamiales fossils and a divergence time between Oleaceae and Antirrhineae modeled as a normal distribution with mean = 74 Ma and Std = 2.5 Ma, on the basis of a relaxed molecular clock analysis of angiosperms [80], see [40] for details. We used the minimum age (13.33 Ma) as a fixed calibration point for the stem node of the *Linaria* clade to estimate the dates of the internal nodes with a penalized likelihood procedure implemented in r8s 1.71 [81]. Cross-validation to find the optimal smoothing parameter (10^k) was done using increments of k of 0.1, from $k = -3$ to 3, repeated for two trees from the stable posterior distribution of each gene; the smoothing values of both trees were very similar so we used the value with lower χ^2 error. After cross-validation we set the smoothing parameter to 1.5 for ITS, 3.2 for AGT1 and 0 for cpDNA and rate smoothed 20 trees drawn from the posterior distribution after burn-in to obtain the chronograms that were used in the coalescent simulations.

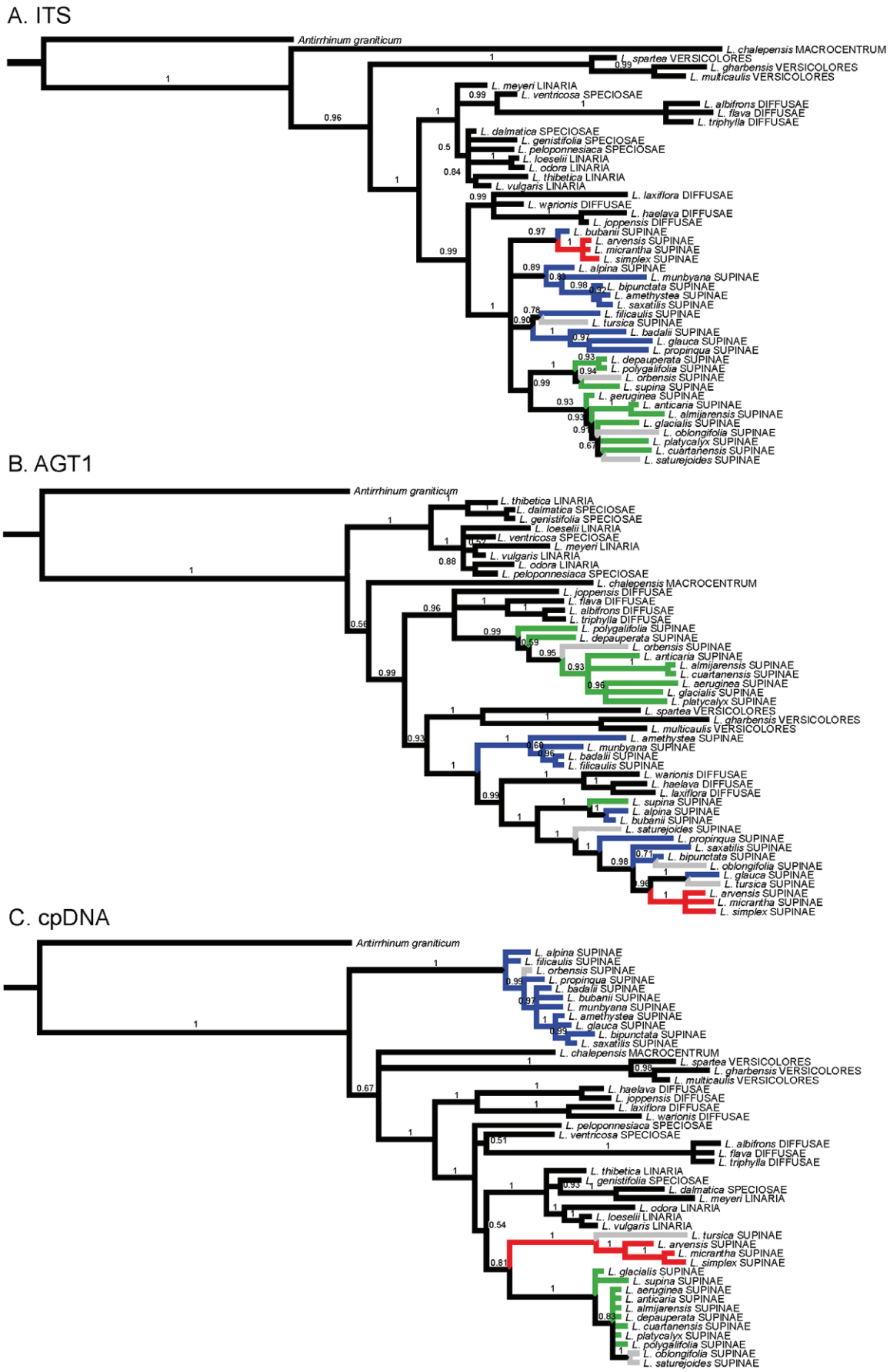
Coalescent Simulations

We used simulations under the coalescent model following Maureira-Butler *et al.* [6] to test whether incomplete lineage sorting alone could explain the observed incongruence among gene trees. As the test does not account for the uncertainty of tree topology and branch length estimation, here we used 20 trees from the stable posterior distribution of the Bayesian analysis for each gene, performed the simulations and calculated all tree-to-tree distances from this pool of trees (hereafter the base line distribution), rather than the consensus as was done previously [6]. The base line distribution was then compared to the distribution obtained by calculating pairwise tree-to-tree distances of the 20 chronograms for each gene –essentially a measure of how much the gene trees from each locus differ– hereafter the observed distribution (see Methods S1 for further details).

Effective population size estimates (N_e) used in the coalescent simulations were derived from cpDNA haplotypes and obtained via $\theta_w = 2\mu N_e$, with theta (θ_w) and mutation rate per generation (μ) taken from data of three *Linaria* species with contrasting range sizes (and potentially, contrasting N_e) (table S2): *L. glacialis* (endangered, narrow endemic of Sierra Nevada, Spain), *L. elegans* (endemic to northern Iberia) and *L. simplex* (distributed across the Mediterranean basin). The effect of N_e estimates in the coalescent simulations was explored by repeating the set of simulations using the three N_e values separately (see Methods S1 for further details).

Detection of Potential Hybrids

The detection of potential hybrids was addressed by examining the effect of taxon deletion on the observed and base line distributions. Theoretically, the potential hybrids detected by the test were the set of individuals that, after exclusion, retrieved overlapping observed distributions (pairwise tree-to-tree distances within their 95% HPD) and base line distributions (trees from coalescent simulations), thus the null hypothesis of incomplete lineage sorting alone was no longer rejected. Here, this approach was difficult to apply as the results were very dependent on the N_e values used (see Results). We identified that limitation, but we also recognized the significant challenge of getting exact estimates of population sizes through time in a phylogeny, especially with scarce genetic data [82,83]. We then made an exploration of the effect of the deletion of each terminal with an incongruent position, in order to identify the individuals causing the highest effect in the differences between the baseline and the observed distributions. This was done by excluding terminals with incongruent positions (one at the time) and calculating new base line and observed distributions for the three datasets under each N_e. The nine replications (three datasets x three N_e) alleviated the non-reproducible effect of taxon exclusion due to the stochastic


A. ITS
Antirrhinum graniticum
L. chalepensis MACROCENTRUM
L. spartea VERSICOLORES
L. gharbensis VERSICOLORES
L. multicaulis VERSICOLORES
L. meyeri LINARIA
L. ventricosa SPECIOSAE
L. albifrons DIFFUSAE
L. flava DIFFUSAE
L. triphylla DIFFUSAE
L. dalmatica SPECIOSAE
L. genistifolia SPECIOSAE
L. peloponnesiaca SPECIOSAE
L. loeselii LINARIA
L. odora LINARIA
L. thibetica LINARIA
L. vulgaris LINARIA
L. laxiflora DIFFUSAE
L. warionis DIFFUSAE
L. haelava DIFFUSAE
L. joppensis DIFFUSAE
L. bubanii SUPINAE
L. arvensis SUPINAE
L. micrantha SUPINAE
L. simplex SUPINAE
L. alpina SUPINAE
L. munbyana SUPINAE
L. bipunctata SUPINAE
L. amethystea SUPINAE
L. saxatilis SUPINAE
L. filicaulis SUPINAE
L. tursica SUPINAE
L. badalii SUPINAE
L. glauca SUPINAE
L. propinqua SUPINAE
L. depauperata SUPINAE
L. polygalifolia SUPINAE
L. orbensis SUPINAE
L. supina SUPINAE
L. aeruginea SUPINAE
L. anticaria SUPINAE
L. almijarensis SUPINAE
L. glacialis SUPINAE
L. oblongifolia SUPINAE
L. platycalyx SUPINAE
L. cuartanensis SUPINAE
L. saturejoides SUPINAE
B. AGT1
Antirrhinum graniticum
L. thibetica LINARIA
L. dalmatica SPECIOSAE
L. genistifolia SPECIOSAE
L. loeselii LINARIA
L. ventricosa SPECIOSAE
L. meyeri LINARIA
L. vulgaris LINARIA
L. odora LINARIA
L. peloponnesiaca SPECIOSAE
L. chalepensis MACROCENTRUM
L. joppensis DIFFUSAE
L. flava DIFFUSAE
L. albifrons DIFFUSAE
L. triphylla DIFFUSAE
L. polygalifolia SUPINAE
L. depauperata SUPINAE
L. orbensis SUPINAE
L. anticaria SUPINAE
L. almijarensis SUPINAE
L. cuartanensis SUPINAE
L. aeruginea SUPINAE
L. glacialis SUPINAE
L. platycalyx SUPINAE
L. spartea VERSICOLORES
L. gharbensis VERSICOLORES
L. multicaulis VERSICOLORES
L. amethystea SUPINAE
L. munbyana SUPINAE
L. badalii SUPINAE
L. filicaulis SUPINAE
L. warionis DIFFUSAE
L. haelava DIFFUSAE
L. laxiflora DIFFUSAE
L. supina SUPINAE
L. alpina SUPINAE
L. bubanii SUPINAE
L. saturejoides SUPINAE
L. propinqua SUPINAE
L. saxatilis SUPINAE
L. bipunctata SUPINAE
L. oblongifolia SUPINAE
L. glauca SUPINAE
L. tursica SUPINAE
L. arvensis SUPINAE
L. micrantha SUPINAE
L. simplex SUPINAE
C. cpDNA
Antirrhinum graniticum
L. alpina SUPINAE
L. filicaulis SUPINAE
L. orbensis SUPINAE
L. propinqua SUPINAE
L. badalii SUPINAE
L. bubanii SUPINAE
L. munbyana SUPINAE
L. amethystea SUPINAE
L. glauca SUPINAE
L. bipunctata SUPINAE
L. saxatilis SUPINAE
L. chalepensis MACROCENTRUM
L. spartea VERSICOLORES
L. gharbensis VERSICOLORES
L. multicaulis VERSICOLORES
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L. odora LINARIA
L. loeselii LINARIA
L. vulgaris LINARIA
L. tursica SUPINAE
L. arvensis SUPINAE
L. micrantha SUPINAE
L. simplex SUPINAE
L. glacialis SUPINAE
L. supina SUPINAE
L. aeruginea SUPINAE
L. anticaria SUPINAE
L. almijarensis SUPINAE
L. depauperata SUPINAE
L. cuartanensis SUPINAE
L. platycalyx SUPINAE
L. polygalifolia SUPINAE
L. oblongifolia SUPINAE
L. saturejoides SUPINAE

Figure 1. Gene trees. Phylogenetic relationships of 47 samples representing 46 *Linaria* species and one individual of *Antirrhinum* as the outgroup. One species of sect. *Macrocentrum*, three species of sect. *Versicolores*, five species of sect *Linaria*, four species of sect. *Speciosae* and 28 species of sect. *Supinae* are represented. 50% Mayority-rule consensus tree obtained in the Bayesian analysis of ITS (A), AGT1 (B) and cpDNA (C) sequences are shown. Numbers above branches represent Bayesian posterior probabilities. Phylogenetic trees are based on one sample and one allele per species, when the two alleles were not sister we used the most incongruent one respecting the other two genes. *Linaria* sections following Sutton [45] are shown in capital letters. Colors represent the systematic nomenclature for *Supinae* clades as suggested in this paper (see Fig. 4). Species with key traits from two *Supinae* clades (Fig. 4) are represented in grey.
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nature of simulations. The last step was to average the nine independent estimates obtained for each analyzed taxon.

Testing Monophyly of *Supinae*

We used AGT1 and cpDNA datasets (one haplotype per sequence) with hybrids excluded to test support for the monophyly of *Supinae*. This was done to assess whether the incongruence (regarding *Supinae* naturalness) was exclusively explained by hybridization (as putative hybrids were excluded) and inference limitations, or whether additional processes generated real gene tree differences (in this case incomplete lineage sorting). In order to calculate support for the monophyly of *Supinae* we used two approaches: (i) the Shimodaira and Hasegawa [84] (S-H) test and the Bayes Factors [85,86] (BF) test. The S-H test was implemented by calculating the maximum likelihood tree with unconstrained and constrained topologies in RAxML (–f d function) to subsequently compare both ML trees using the –f g function, which computes the per-site log Likelihoods for the contrasted topologies. The per-site log Likelihoods were analyzed with CONSEL [87] to obtain the S-H statistic values. BF test was used to assess alternative phylogenetic hypothesis in a Bayesian framework [85,86]. The BF test quantifies the support for one hypothesis versus another given the data. We also used this approach, implemented in Tracer 1.4 [88] to test significant differences between the unconstrained and constrained Bayesian analyses of AGT1 and cpDNA. Stationarity and convergence of analyses were assessed in Tracer after discarding the first 10% of sampled generations as burn-in. Marginal likelihoods, their standard errors (estimated using 1000 bootstrap replicates) and BFs were calculated. We considered 2xlnBF(H_1 vs. H_0) -2 to -6 as positive evidence against H_1 *in favor of* H_0; 2xlnBF(H_1 vs. H_0) -6 to -10 as strong evidence against H_1 *in favor of* H_0; and 2xlnBF(H_1 vs. H_0) <-10 as very strong evidence against H_1 *in favor of* H_0 [89].

Species Tree Inference

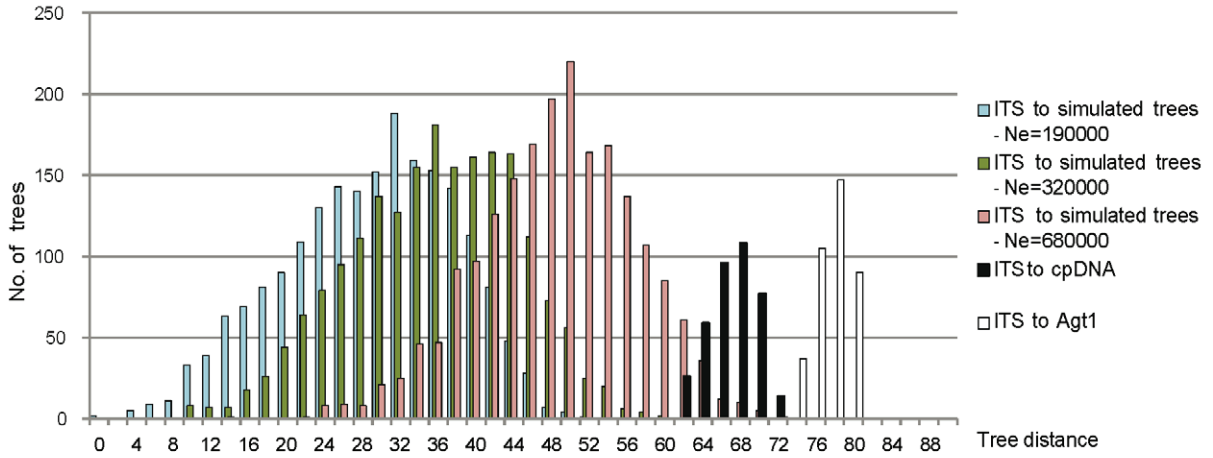
After excluding potential hybrids (to not violate the species tree model assumptions), we used the allelic data (and >1 individual per species in some cases, see Table S1) to estimate the species tree with the *BEAST (StarBeast) method [20] implemented in BEAST v.1.6.2. [89]. Allelic data were included in three data partitions with unlinked genealogies: (i) ITS sequences, (ii) AGT1 sequences and (iii) combined plastid (*rpl32-trnL*^{UAG} and *trnS-trnG*) sequences. We used Sutton's species delimitation [45], but additionally recognizing *L. almijarensis* Campo & Amo [64] (one population). The prior probability of the divergence time between *Linaria* and *Antirrhinum* was constrained to 20 Ma ± 4 as a normal distribution, following date estimates obtained for the tribe Antirrhineae (Vargas *et al.*, unpublished, see "Gene trees estimation and calculation of dates" section). A Birth-Death process [90] was employed as the species tree branching prior. We used an uncorrelated lognormal relaxed clock model, with the prior probability for the substitution rate uniformly distributed, with ranges of 5×10^{-4}-5×10^{-2} and 1×10^{-4}-1×10^{-2} substitutions per site per Ma (s/s/Ma) for the nuclear loci and the plastid locus respectively. These rate constraints include previous estimates for herbaceous plant ITS rates ($1.7–8.3\times10^{-3}$ s/s/Ma) [91] and chloroplast rates ($1.0–3.0\times10^{-3}$ s/s/Ma) [92]. Nuclear synonymous substitution rates, being nearly neutral, may approximate nuclear intron rates. The former rates have been found in other plants to lie within the range we used (e.g., 48 *Gossypium* genes, $3.5–7.3\times10^{-3}$ s/s/Ma, [93]; 39 legume genes, mean of 5.2×10^{-3} s/s/Ma, [94]). Six MCMC analyses were run for 30 million generations each, with a sample frequency of 1000. Analysis with Tracer v.1.5 [88] confirmed convergence of analyses and adequate sample sizes, with ESS values above 200. Analyses were combined using LogCombiner v.1.6.2 after discarding the first 10% generations of each run as burn-in. Trees were summarized in a maximum clade credibility tree using TreeAnotator v.1.6.2. After combination of the six log files from the analyses, the standard deviation of the uncorrelated lognormal relaxed clock (ucld.stdev) and the coefficient of variation (CoV) in the three genes were not close to 0: cpDNA ucld.stdev = 0.94, cpDNA Cov = 0.97; AGT1 ucld.stdev = 0.806, AGT1 CoV = 0.854; ITS ucld.stdev = 0.685, ITS CoV = 0.702. This branch rate heterogeneity indicated that the uncorrelated lognormal relaxed clock was appropriate.

Multilabelled Species Tree

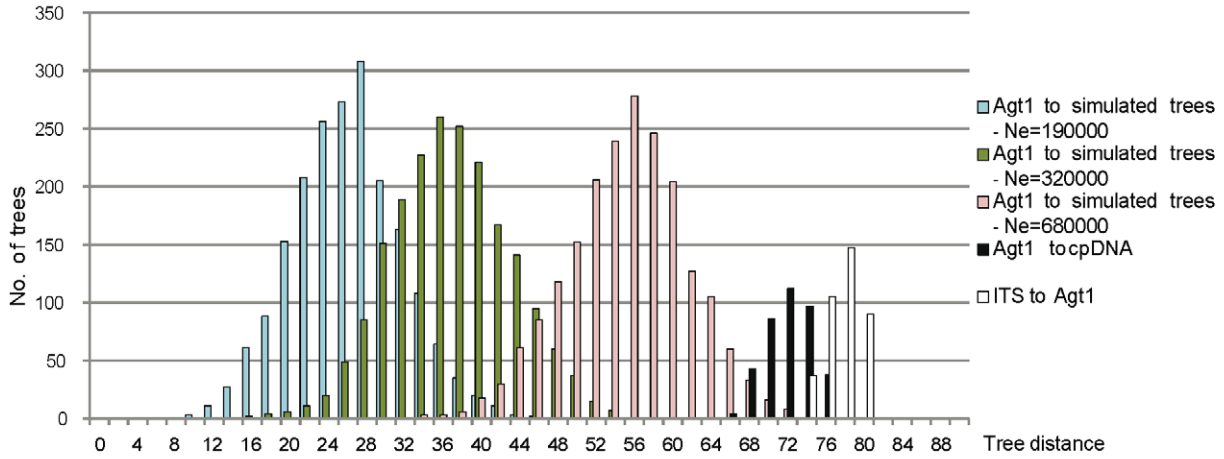
A multilabelled species tree was inferred to retrieve the origin of the parental lineages of individuals affected by reticulation processes. We inferred a second species tree but this time including allelic data from potential hybrids. We recalculated the best-fitting model of sequence evolution with jModeltest 0.1.1 [78,79], while the remaining priors were set as in the species tree analysis. The multilabelled species tree was built by assigning the two most congruent genes to one label (tip, or terminal species branch) and the remaining gene to a second label (see Table S3) while using missing data for the gene not assigned in the label. Thus, the two labels of a potential hybrid species (L1 and L2) where treated as different "species" in *BEAST analysis in order to show which two hybridizing lineages have contributed to a lineage of hybrid origin. The analysis therefore treated the differences between the two most congruent genes as being caused by incomplete lineage sorting alone, whereas our multilabelling approach allowed the differences between the most incongruent positions to be due to hybridization without violating the assumptions of the *BEAST model. The key concept is that a lineage of hybrid origin has two sources of parental contribution to its genome. These origins are best represented in a tree diagram by including two labels rather than just one (as is the case for lineages without a hybrid origin). This approach is novel, as far as we know, but has similarities to the approach used by Pirie *et al.* [95]. Four MCMC analyses were run for 100 million generations each, with a sample frequency of 10000. Analysis with Tracer v.1.5 [88] also confirmed convergence of analyses and adequate sample size, with ESS values above 200. We combined the analyses and summarized the tree as indicated above.

In order to contrast the results of the multilabelled species tree with other procedures widely used in phylogenetic studies, we also

A. ITS



B. AGT1



C. cpDNA

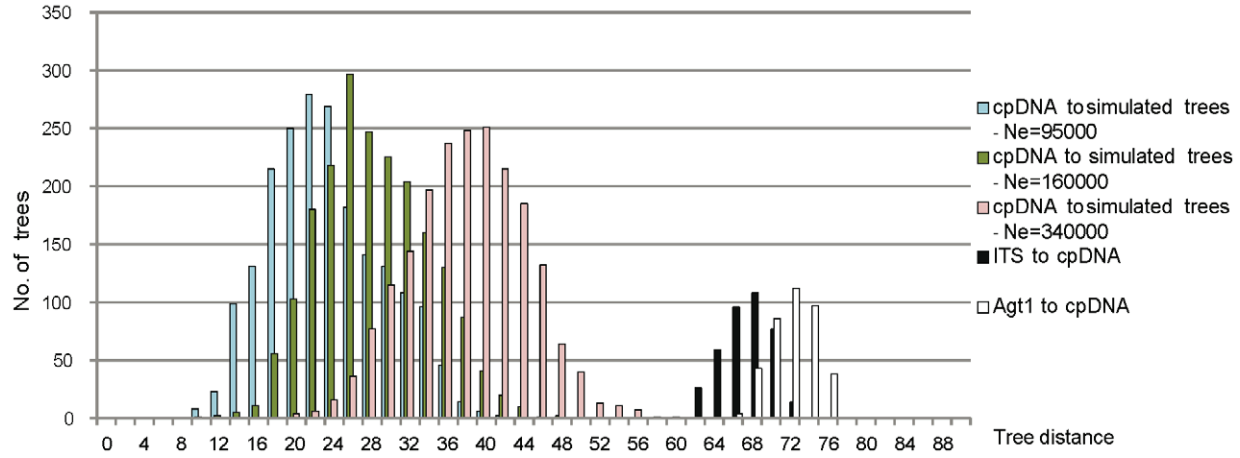


Figure 2. Baseline and observed distributions of tree distances. Frequency distribution of tree-to-tree distances between 20 representative trees from the stable posterior distribution of the Bayesian analysis (ITS (A), AGT1 (B) and cpDNA (C)) and 100 simulated gene trees obtained by coalescent simulations (baseline distributions). Blue, green and red bars represent baseline distributions under *L. glacialis*, *L. elegans* and *L. simplex* N_e estimates respectively. Black and white bars represent the distances between gene trees (observed distributions).
doi:10.1371/journal.pone.0039089.g002

performed a *BEAST species tree analysis and a total evidence analysis, both with potential hybrids included.

Results

Haplotype Data

The Arlequin analysis gave us the two most probable haplotypes from the unphased genotypes of AGT1 and ITS sequences. For AGT1, we obtained haplotypes of 50 individuals with posterior probabilities (PP) above 0.95 and haplotypes of four individuals with PP below 0.95. For ITS, we obtained haplotypes of 34 individuals with PP above 0.95 and haplotypes of 20 individuals with PP below 0.95. The AGT1 phased data retrieved for the four individuals with low PP were empirically confirmed by amplicon cloning, recovering exactly the same allelic data that Arlequin inferred. As ITS is a multi-copy locus marker, there would be more than two copies for each unphased ITS genotype. This may have affected the haplotype detection, thus giving low support for the ITS haplotypes obtained. But (i) as one haplotype with low probability and differential position in the ITS allele-tree has been confirmed empirically (*L. bubanii*, 0.41 PP) and (ii) highly differentiated alleles have not been obtained in the Arlequin analyses (excluding *L. bubanii*), being all sister or closely-related in allelic-gene trees, we then considered that the two ITS haplotypes detected by Arlequin were good representatives of the existing ITS alleles per sample.

Recombination Test

Recombination could not be detected in ITS by any of the five methods used. AGT1 showed one recombination event affecting several sequences that was detected by SiScan (Av. p-value = 3.712×10^{-2}) but when contrasting the UPGMA trees of the recombinant and non-recombinant regions it showed almost the same topology with both potential parents separated in the tree from the potential recombinants. Additionally, evidence for recombination was not considered convincing if it only was

Table 2. Effect of taxa exclusion on the differences between base line (from simulated trees) and observed distributions of tree distances, numbers indicate steps while negative (-) and positive (+) values indicate approximation and separation between distributions, respectively.

	Effect after taxa deletion (steps)									
	ITS baseline distribution to ITS-cpDNA observed distribution			**AGT1 baseline distribution to AGT1-cpDNA observed distribution**			**cpDNA baseline distribution to ITS-cpDNA observed distribution**			
Taxa with incongruent position in gene trees	**A**	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**	**Average effect**
L. glauca ssp. *olcadium**	−4§	−2	−4	−3	−2	−2	−2	−2	−2	*−2.56*
*L. orbensis**	−4	0	−4	+1	+1	+1	−2	−2	−2	*−1.22*
L. amethystea ssp. *amethystea**	−3	−1	−3	−1	0	+1	−1	−1	−1	*−1.11*
*L. cuartanensis**	+1	−1	−1	−1	−1	−1	−1	−2	−2	*−1.00*
*L. tursica**	+2	+2	0	−2	−2	−1	−2	−2	0	*−0.56*
L. oblongifolia ssp. *oblongifolia**	0	0	0	−2	−1	−1	0	0	0	*−0.44*
*L. alpina**	0	+2	0	−1	−1	+1	0	−2	−2	*−0.33*
*L. filicaulis**	0	0	0	−1	−1	−1	0	0	0	*−0.33*
L. saturejoides ssp. *saturejoides**	0	0	0	−2	0	0	0	0	0	*−0.22*
*L. propinqua**	−2	+1	−2	−1	+1	+1	0	0	0	*−0.22*
L. supina ssp. *supina*	0	+2	−2	−1	+1	+1	0	0	0	*+0.11*
L. bubanii	0	+2	−2	+1	−1	+1	0	−1	+1	*+0.11*
L. bipunctata ssp. *bipunctata*	−2	+2	0	−1	0	0	0	+2	0	*+0.11*
L. saxatlis	−2	+3	−2	+1	0	+1	0	0	0	*+0.11*
L. badalii	−1	+3	−1	−1	−1	−1	+1	+1	+1	*+0.11*
L. almijarensis	+1	+3	+1	−1	−1	−1	+1	0	0	*+0.33*
L. mumbyana	+1	+3	+1	−1	0	−1	+1	0	0	*+0.44*
L. aeruginea	+1	+1	+1	0	+1	+1	0	−1	0	*+0.44*

***A L. glacialis* N_e:** Nuclear $N_e = 190000$, Plastid $N_e = 95000$.
***B L. elegans* N_e:** Nuclear $N_e = 320000$, Plastid $N_e = 160000$.
***C L. simplex* N_e,** Nuclear $N_e = 680000$, Plastid $N_e = 340000$.
*Individuals of putative hybrid origin that were excluded from the analysis in Figure 4.
§Calculation plotted as an example in Fig. 3.
doi:10.1371/journal.pone.0039089.t002

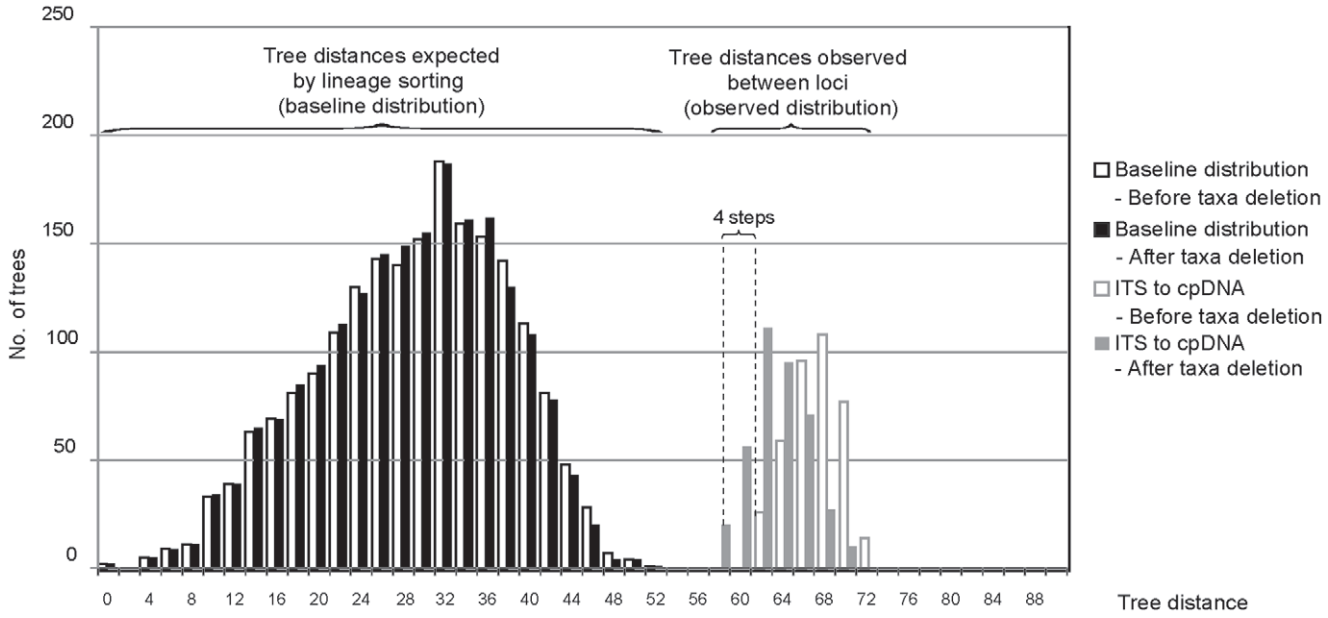


Figure 3. Detection of potential hybrids. An example illustrating the method used for the detection of potential hybrids. It is shown the effect of the exclusion of *L. glauca* ssp. *olcadium* on the differences between base line and observed distributions of tree distances.
doi:10.1371/journal.pone.0039089.g003

detected by a single method as done in Poke *et al.* [96]. Therefore, we proceeded without removing the sequences under discussion.

Gene Tree Inference

ITS phylogenetic analysis supported monophyly for section *Supinae* sister to a group formed by four species of sect. *Diffusae* (*L. laxiflora*, *L. warionis*, *L. haelava*, *L. joppensis*). In ITS, relationships within *Supinae* were not clearly related to morphological features (Fig. 1A). The AGT1 region did not support monophyly of the section, as species of sect. *Diffusae* and sect. *Versicolores* were grouped together with sect. *Supinae*. The three *Supinae* groups detected in AGT1 were also not obviously correlated with morphological characters (Fig. 1B). The cpDNA dataset did not support monophyly of the section, as there were two clearly separated groups of *Supinae* species, however, this locus showed three well-supported groups within *Supinae* associated with corolla sizes and seed shape (Fig. 1C).

Coalescent Simulations

When using the small and medium (*L. glacialis* and *L. elegans*, respectively) N_e estimates (Table S2), the pairwise distances of gene trees lay outside the base line distribution for either gene (Fig. 2). Contrastingly, when using the largest N_e values (from widespread *L. simplex*), the pairwise distances of gene trees lay inside the base line distribution of ITS and AGT1 genes (Fig. 2). As we expected a high overestimation of the population size when using *L. simplex* N_e, these results reflected that the degree of incongruence in the three gene trees was difficult to explain by incomplete lineage sorting alone when applying Maureira-Butler's test [6].

Detection of Potential Hybrids

When using simulations obtained with medium N_e values (*L. elegans*), only one individual needed to be removed in order to retrieve overlapping baseline and observed distributions (not shown), and therefore only one potential hybrid could be considered robustly detected. In contrast, when using simulations

Table 3. Results of Shimodaira-Hasegawa (S-H) test and Bayes Factors (BF) test with observed log-likelihood difference obtained in Maximum Likelihood analyses, S-H test statistics, mean values of marginal likelihood of the Bayesian analyses and BF test statistics (2xlnBF) for the unconstrained analysis (H_0) and the analysis with monophyly of *Supinae* constrained in AGT1 and cpDNA datasets (H_1).

Gene tree	Hypothesis (H)	S-H test		BF test	
		Observed log-likelihood difference	SH statistic	Marginal likelihood (lnP(model | data)) ± SE	2xlnBF (H vs. H0)
AGT1	H_0	–	–	−3513.183±0.27	–
	H_1: monophyly of sect. *Supinae*	35.9	0.01*	−3547.955±0.33	−69.546**
cpDNA	H_0	–	–	−4461.125±0.27	–
	H_1: monophyly of sect. *Supinae*	35.4	0.01*	−4488.746±0.26	−55.242**

*≤0.05, support for rejection of H_1.
**≤10, very strong evidence for rejection of H_1.
doi:10.1371/journal.pone.0039089.t003

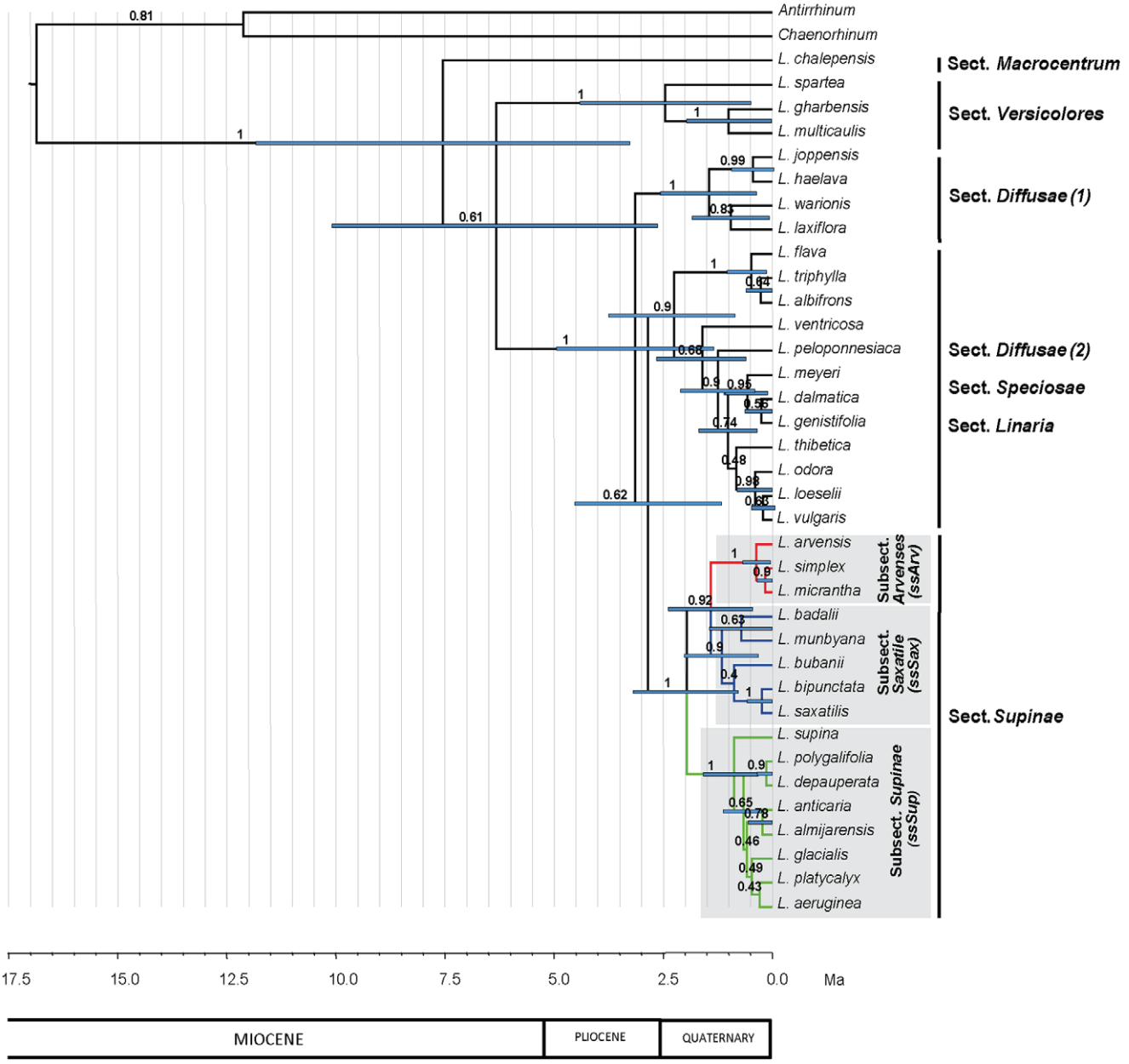


Figure 4. Species tree of *Linaria*. Maximum clade credibility tree obtained in the *BEAST species tree analysis after excluding potential hybrids and using allelic data of ITS, AGT1 and cpDNA datasets. Node bars represent the 95% highest posterior density intervals for the divergence time estimates of nodes with posterior probabilities above 0.50. Values above branches indicate Bayesian posterior probabilities. *Linaria* sections following Sutton (1988) are shown. Colors and clade labels represent the systematic nomenclature for *Supinae* as suggested in this paper.
doi:10.1371/journal.pone.0039089.g004

Table 4. Morphological key traits of the subsections proposed for section *Supinae* regarding the results obtained in the *BEAST species tree analysis of ITS, AGT1 and cpDNA sequences (Figure 4).

	Subsect. *Arvenses*	**Subsect. *Saxatile***	**Subsect. *Supinae***
Corolla size	**Small (2.5–9 mm)**	**Medium (6–18 mm)**	**Large (16–31 mm)**
Seed wing shape	Thick-wide	Thick-wide/narrow (or absent)	Membranous-wide
Life-form	Annual	Annual/Perennial	Perennial

doi:10.1371/journal.pone.0039089.t004

Table 5. Divergence dates of clades of *Linaria* sect *Supinae*, presented as mean crown ages and 95% highest posterior density (HPD) intervals based on the *BEAST species tree analysis (Figure 4).

Clade/Lineage	Mean age of divergence (Ma)	95% HPD interval
Genus *Linaria*	7.55	3.57–12.14
Sect. *Supinae*	1.97	0.87–3.28
Sect. *Supinae* subsect. *Arvenses*	0.36	0.08–0.72
Sect. *Supinae* subsect. *Saxatile*	1.16	0.39–2.08
Sect. *Supinae* subsect. *Supinae*	0.87	0.31–1.58

doi:10.1371/journal.pone.0039089.t005

with the smallest N_e values (*L. glacialis*), even after removing all the individuals with incongruent positions, we still had non-overlapping distributions (not shown), and consequently all species with incongruent positions (17 spp.) were identified as potential hybrids. Therefore, our N_e estimates showed all possible scenarios: (i) gene tree incongruence is explained by incomplete lineage sorting alone (*L. simplex* N_e), (ii) gene tree incongruence is explained by both incomplete lineage sorting and hybridization (*L. elegans* N_e) and (iii) gene tree incongruence is explained by hybridization alone (*L. glacialis* N_e). These results clearly illustrated the high dependence on N_e estimates in order to obtain the exact number of individuals of hybrid origin. We assumed that a reliable number of potential hybrids lay between the two extreme values obtained in (ii) and (iii).

The effect of the deletion of each incongruent individual on both the observed and base line distributions is shown in Table 2. We considered that individuals with the highest probability of hybrid origin were those individuals that, after deletion, decreased (on average) the differences between the base line and the observed distributions (in number of steps, see an example in Fig. 3). Ten of the 17 incongruent individuals decreased the differences among distributions and consequently were considered to be potential hybrids or to have a hybrid history in the broadest sense.

Testing Monophyly of *Supinae*

After excluding putative hybrids, the S-H tests indicated that the constrained topologies for AGT1 and cpDNA had significantly worse likelihood scores than the unconstrained topologies (Table 3), thus monophyly of *Supinae* for these genes was statistically rejected. The BF test (Table 3) also recovered decisive (very strong) support ($2\text{xlnBF} < -10$) for rejection of monophyly of *Supinae* in the AGT1 and cpDNA. As monophyly of *Supinae* was recovered in ITS (Fig. 1A), topological incongruence in concert with S-H and BF test suggested that processes other than hybridization and inference limitations were also responsible for the topological incongruence among genes.

Species Tree Inference

The *BEAST species tree analysis (potential hybrids excluded) (Fig. 4) retrieved four well supported groups within *Linaria*: (i) sect. *Versicolores* (1 PP), (ii) four species of sect. *Diffusae* (1 PP), (iii) a group formed by: three species of sect. *Diffusae*, four species of sect. *Speciosae* and five species of sect. *Linaria* (0.9 PP); and (iv) all sect. *Supinae* species (1 PP). Therefore sect. *Supinae* was retrieved as a monophyletic group with high support and was divided in three clades: one clade was represented by three annual species (*L. arvensis, L. simplex, L. micrantha*; 1 PP) with small corollas (2.5–9 mm) and a thick-wide seed wing (subsect. *Arvenses,* hereafter *ssArv*). A second clade was represented by five annual or perennial species (*L. badalii, L. munbyana, L. bubanii, L. bipunctata, L. saxatilis*; 0.90 PP) with medium-sized corollas (6–18 mm) and a thick-wide seed wing or narrow wing (marginal ridge) (subsect. *Saxatile,* hereafter *ssSax*). The third clade contained eight perennial species (*L. supina, L. polygalifolia, L. depauperata, L. anticaria, L. almijarensis, L. glacialis, L. platycalyx, L. aeruginea*; 1 PP) with large corollas (16–31 mm) and a membranous-wide seed wing (subsect *Supinae,* hereafter *ssSup*) (see Table 4).

The * BEAST species tree detected that incomplete lineage sorting has affected all gene trees analyzed. In the ITS dataset we detected deep coalescence at medium depth branches (see *L. bubanii* position in the ITS tree and *BEAST species tree); from the AGT1 dataset we detected deep coalescence at medium depth branches (*L. munbyana, L. badalii*) and at deeper branches (*L. polygalifolia, L. depauperata, L. orbensis, L. anticaria, L. almijarensis, L. aeruginea, L.glacialis* and *L. platycayx*); in cpDNA we also detected deep coalescence at the deepest branches (*L. badalii, L. bubanii, L. munbyana, L. bipuncata* and *L. saxatilis*).

The time to the most recent common ancestor (TMRCA) of *Supinae* was placed in the late Pliocene-early Pleistocene (0.87–3.28 Ma), the TMRCA of *ssArv* was located in the middle-late Pleistocene (Ionian-Tarantian) (0.08–0.72 Ma), the TMRCA of *ssSax* in the early-middle Pleistocene (Gelasian-Calabrian-Ionian) (0.39–2.08 Ma) and the TMRCA of *ssSup* in the early-middle Pleistocene (Calabrian-Ionian) (0.31–1.58 Ma) (see Table 5).

Multilabelled Species Tree

The multilabelled species tree (Fig. 5) retrieved a well supported clade (0.96 PP, *ssSup*) and a clade with moderate support (0.89 PP, *ssSax+ssArv*) within *Supinae*. Out of ten reticulation events that have been presumed to occur, one was produced within the *ssSup* clade, six within the *ssSax+ssArv* clade and three between these two clades. One of the six potential hybridization events within ssSax+ssArv clade is reflected in *L. tursica*, a species with morphological traits typical from both ssSax and ssArv clades (Fig. 4): wingless seed (some species of ssSax present narrow to marginal seed wings) and small corolla (ssArv). The three reticulation events inferred between ssSup and ssSax+ssArv produced three species with morphological traits typical of both clades (*L. orbensis, L. saturejoides and L. oblongifolia*, see Fig. 5 and Table 6).

We estimated the timing of the hybridization events by looking at the divergence time of parental lineages of putative hybrids. As hybridization could not take place prior to divergence of parental lineages, divergence time for the most recent lineage constituted the maximum age of each hybridization event. Despite the topological uncertainty at the tips, we found that all bar one maximum age of the presumed hybridization episodes occurred during the Pleistocene (Fig. 5 and Table 7). In a single case, *L. tursica*, the 95% HPD overlapped the Middle Pliocene, although the mean estimate remained within the Pleistocene (Table 7).

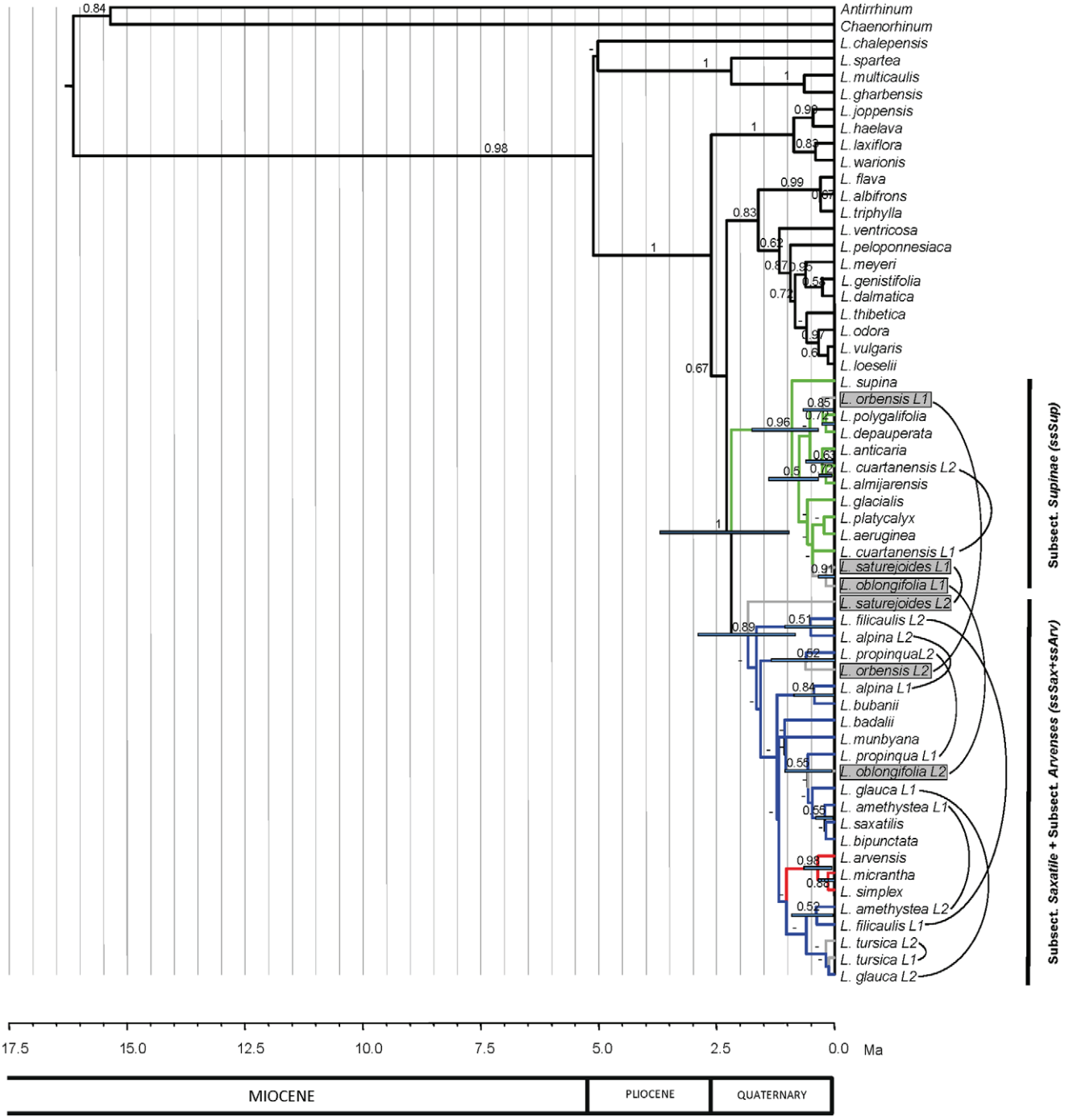


Figure 5. Reticulate evolution in *Supinae*. Maximum clade credibility tree obtained in the multilabelled *BEAST species tree analysis by including the presumed hybrids connected in two labels (L1 and L2) representing the two parental lineages of hybrid species. Node bars represent the 95% highest posterior density intervals for the divergence time estimates of nodes with posterior probabilities above 0.50 (only divergence time estimates for *Supinae* lineages are shown). Values above branches indicate Bayesian posterior probabilities. A hyphen (-) indicates posterior probability below 0.50. Colors and tree labels represent the systematic nomenclature for *Supinae* as established in this paper. Species labels of putative hybrids produced by the cross of the two main *Supinae* clades are highlighted in grey.
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Discussion

Using a Coalescent Framework to Disclose the Evolutionary History of *Supinae*

Systematics of *Linaria* and specifically sect. *Supinae* has been subject to various interpretations in numerous taxonomic treatments in the last two centuries. Historical disagreement occurred when discerning the naturalness of the section and its internal classification [43–51] (see Table 1). To disclose the evolutionary history of *Supinae*, we sampled genetic data from 46 *Linaria* species, including sequences from three presumably unlinked genes. Because of the highly supported incongruence among trees based

Table 6. Morphological key traits of species with putative hybrid origin produced by the cross between subsect *Saxatile* + subsect *Arvenses* (*ssSax+ssArv*) and subsect *Supinae* (*ssSup*) parental lineages based on the results obtained in the *BEAST multilabelled species tree analysis (Figure 5).

	L. orbensis	*L. saturejoides*	*L. oblongifolia*
Corolla size	**Medium (11–15 mm)**	**Medium (12–17 mm)**	**Medium-large (15–22 mm)**
Seed wing shape	Membranous-wide	Membranous-wide	Membranous-wide
Life-form	Annual	Annual	Annual

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on separate analysis of the three genes, difficulty in the systematic reconstruction of *Supinae* at this stage of analysis was patent, the naturalness of the section remained unclear and the infra-sectional classification was still controversial.

In the last few years the incorporation of the coalescent model into phylogenetic analysis has greatly improved the theoretical basis for inferring species trees from gene trees via a mixed model –the multispecies coalescent (e.g., BEST [28]; *BEAST [20]; [97]). One key practical challenge is to include only data that meet the assumptions of the current implementations. Of significant concern is to properly handle sequences, individuals or taxa with multiple histories, such as by excluding recombinants or hybrids prior to species tree inference.

Here, we performed simulations under coalescence following the method of Maureira-Butler *et al.* [6] to estimate whether the gene tree incongruence detected among genes could be explained by incomplete lineage sorting without hybridization. The test exposed that with small and medium N_e values used in the simulations, the topological variation generated by incomplete lineage sorting was not as high as the incongruence observed between the three genes (Fig. 2), whereas with high N_e (*L. simplex* N_e), the variation generated by incomplete lineage sorting alone could explain the totality of incongruence observed between genes (Fig. 2). We considered that the high N_e greatly overestimated the general N_e of *Linaria*, as only 9 out of 150 *Linaria* species have a similar wide range size [45] (and presumably similar N_e). Hence, the results of Maureira-Butler's test suggested that incongruence among genes was difficult to explain by incomplete lineage sorting alone, indicating that hybridization may also account for the gene tree inconsistency. However, the exact number and identity of individuals that may have hybrid histories is not clearly established here, because of the sensitivity of the test to N_e estimation. We consider, instead, that the test has provided a probable set of individuals that may adversely affect the *BEAST analysis and that a cautious approach (removing these individuals before the analysis) is preferred here, rather than risking a spurious species tree inference.

The hybrid detection test (Table 2) and the multilabelled *BEAST species tree (Fig 5) was also contrasted with a *BEAST species tree including all potential hybrids (not shown). After six runs with 30 million generations, convergence could not be reached and some ESS values (of population size parameters) remained under 200, which illustrated that the inclusion of potential hybrids may be violating assumptions of the *BEAST analysis. Our approach was also contrasted with an additional analysis of the three datasets concatenated in a total evidence approach (see Fig. S1). Results of both approaches (our multilabelled species tree with hybrids excluded vs. the total evidence analysis) gave highly conflicting results. These discordant results were expected, as it is known that concatenation of data from multiple loci may lead to biased phylogenetic estimates under widespread incomplete lineage sorting and/or hybridization [19]. Results presented here highlight the paramount importance of (i) analyzing multiple loci datasets in a multispecies coalescent approach in order to find a more realistic species tree and (ii) the requirement of additional analytical tools to identify and to disclose the origin of species affected by historical hybridization. We note that our multilabelled species tree still allows the possibility of observing congruent placements for each label of the same individual. That is, we are not forcing different placements with this approach, but instead allowing them, if preferred by the data. Therefore, this

Table 7. Divergence dates of parental lineages of hybrid species presented as mean age of divergence and 95% highest posterior density (HPD) intervals based on the *BEAST multilabelled species tree analysis (Figure 5).

Hybrid taxa	Mean age of divergence from most recent parental lineage (Ma)	95% HPD interval	Mean age of divergence from 2nd parental lineage (Ma)	95% HPD interval
L. glauca ssp. olcadium	0.61	0.17–1.17	1.53	0.62–2.68
L. orbensis	0.29	0.03–0.67	0.51	0.00–1.35
L. amethystea ssp. amethystea	0.17	0.02–0.38	0.36	0.00–0.89
L. cuartanensis	0.10	0.00–0.28	0.61	0.23–1.09
L. tursica	1.53	0.62–2.68	1.53	0.62–2.68
L. oblongifolia ssp. oblongifolia	0.12	0.00–0.36	0.61	0.17–1.17
L. alpina	0.35	0.00–0.86	0.35	0.00–1.04
L. filicaulis	0.35	0.00–1.04	0.36	0.00–0.89
L. saturejoides ssp. *saturejoides*	0.12	0.00–0.36	1.53	0.62–2.68
L. propinqua	0.51	0.00–1.35	0.61	0.17–1.17

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Table 8. Previous phylogenetic studies of Mediterranean plants with highly supported incongruence among gene trees, we indicate those articles that claim hybridization and/or incomplete lineage sorting as major causes of topological inconsistency.

	Suggested cause of incongruence		
Mediterranean plant group	**Hybridization**	**Incomplete lineage sorting**	**Reference**
Antirrhinum	✓	✗	[102]
Euphorbia sect. *Aphyllis*	✓	✗	[110]
Anthemis	✓	✗	[111]
Centaurium	✓	✗	[112]
Heliosperma	✓	✗	[11]
Reseda sect. *Glaucoreseda*	✗	✓	[100]
Ptilostemon	✗	✓	[113]
Hordeum	✗	✓	[114]
Amarillidaceae (Mediterranean clade)	✗	✓	[115]
Achillea	✓	✓	[116]
Senecio sect. *Senecio*	✓	✓	[117]
Arenaria sect. *Plinthine*	✓	✓	[118]
Phlomis crinita/lychnitis complex	✓	✓	[119]
Medicago	✓	✓*	[6]

*Although not explicitly discussed, incongruence due to incomplete lineage sorting is also apparent among gene trees in this paper.
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approach appears to combine the ideals of utilizing the available comparable data sets (including hybrids) while also appropriately accommodating processes that may cause incongruence (incomplete lineage sorting) and could otherwise lead to spurious tree inference.

Systematics and Drivers of Evolution in *Supinae*

The *Linaria* *BEAST species tree retrieved three well supported clades that agreed with previous classifications (Fig 4): (i) Sect. *Versicolores*, (ii) four species of Sect. *Diffusae* and (iii) Sect. *Supinae*. It also retrieved a group that was incongruent with previous taxonomic treatments. This latter group contained three species of Sect. *Diffusae*, four species of Sect. *Speciosae* and five species of Sect. *Linaria*. In this analysis *Supinae* was monophyletic, as found in the ITS phylogeny. Furthermore, *Supinae* was divided into three morphologically-based subclades consistent with life-form, corolla size and seed wing shape (Table 4), as found in the cpDNA phylogeny: subsect. *Supinae* (*ssSup*), subsect. *Arvenses* (*ssArv*) and subsect. *Saxatile* (*ssSax*). These results are strikingly consistent with some earlier hypotheses, despite the incongruence observed among gene trees. *ssSup* contained eight species that were grouped together in several previous morphological classifications, *ssArv* contained three species that were also previously grouped in a taxonomical entity, whereas *ssSax* contained five species that were historically placed in several distinct taxonomic groups (Systematic proposal in Table 1, diagnostic characters in Table 4). Corolla size and seed wing shape were also previously used as diagnostic characters in a morphological taxonomic revision of winged-seeded *Linaria* species [46]. This author considered *Arvenses (ssArv)* (small flowers) as an independent section and divided *Supinae* in three subsections according to life form and seed wing shape: (i) subsect. *Supinae (ssSup)*: perennial plants with membranous seed wings, (ii) subsect. *Amethystea*: annual plants with thick seed wings and (iii) subsect. *Saxatile:* annual or perennial plants with somewhat thin wings.

Reproductive biology and interaction with pollinators may have played an important role in differentiation within *Supinae*. This is supported by the fact that the species with very low investment in flower structures (small corollas, *ssArv*) are all self-compatible, whereas species with a high investment in flower formation (large corollas, *ssSup*) are all self-incompatible, mainly pollinated by large bees and with low pollinator diversity (Blanco-Pastor & Vargas, unpublished). Geography appears to have played a role in structuring the diversity within *Supinae* as the diversity of *ssSax* is located in the northern part of the Iberian Peninsula (three out of the five species are northern Iberian endemics), whereas the diversity of *ssSup* is located in southern Iberia (five out of eight species are southern Iberian endemics). The timing of divergence of the three subclades (crown nodes, Table 5) indicates that diversification occurred during the Quaternary, after the establishment of the Mediterranean climate regime [39], when species had to tolerate the climatic oscillations occurring in that period [98,99]. This pattern of geographical differentiation driven by Quaternary interglacial fragmentation has been previously identified in many Iberian plants [36,37,100], including the closely-related genus *Antirrhinum* [101,102].

Hybridization during the Quaternary Glaciations

We found that historical hybridization has been likely during the course of *Supinae* evolution. Our analyses identified 10 out of 17 individuals with incongruent positions in gene trees that were difficult to reconcile with incomplete lineage sorting (Table 2). Simple introgression (that is, recurrent horizontal gene flow toward one parental species without formation of new species) can explain the observed gene tree incongruence in those individuals. But the observed pattern could have been also generated by homoploid hybrid speciation (all *Linaria* species analyzed here are diploid (2n = 12) excluding *L. chalepensis* (2n = 24)). Despite speciation via homoploid hybridization has been historically hard to detect (as it could present a similar signal to simple introgression or incomplete lineage sorting) [31], recent studies have suggested that it might be an important mechanism for plant speciation [58,103,104]. Our analyses do not validate speciation via homoploid hybridization, but this process must not be discarded as potential generator of diversity in *Supinae*.

The multilabelled *BEAST species tree analysis (Fig. 5) recovered, to some degree, the origin of the parental alleles of

individuals affected by historical hybridization. There is bound to be a loss of power, because of the reduced number of loci available to place the multilabelled species as well as the need to use missing data. Even so, out of ten potential hybridization events detected, our analyses suggested that one occurred within the *ssSup* lineage, three between two distant parental lineages (*ssSax+ssArv* and *ssSup*) and six within the *ssSax+ssArv* lineage. Crosses between the two distant parental lineages retrieved in the analysis (*ssSax+ssArv* and *ssSup*) were also supported by morphology, given that those three taxa (*L. orbensis*, *L. oblongifolia* and *L. saturejoides*) presented morphological key traits from both clades (Table 6). All hybridization events inferred here were also supported by the results obtained in experimental crosses performed by Valdés [53]. In that study, this author obtained fruits in one of the four crosses performed among *ssSup* species, three of the four crosses between *ssSax+ssArv* and *ssSup* species and four of the seven crosses among *ssSax+ssArv* species (note that here we only accounted for crosses produced between species used in this study thus a higher number of total successful crosses were produced, see [53]).

The maximum age of a hybridization event was considered here to be the maximum age of the origin of the most recent parental lineage. Those ages were circumscribed between 0.28–1.35 Ma in nine of the ten potential hybrids (Table 7). Only in *L. tursica* did the maximum age of hybridization surpass 2.5 Ma (2.68 Ma). Taking into account the effect of low phylogenetic resolution that obscured the detection of ages in parental lineages (thus considering the maximum age of hybridization at deeper nodes), the present results lead us to affirm that all potential hybridization events detected but one may have occurred during the Pleistocene climatic oscillations. During the Quaternary, hybrid zones were established in contact zones (Pyrenees, Alps, Central Europe and Scandinavia) of interglacial northward colonization routes from the temperate regions of Europe [105,106]. In the Iberian Peninsula, where ice effects were less severe, subsequent patterns of contraction, fragmentation, persistence, expansion and admixture during altitudinal migrations may have repeatedly produced multiple hybrid zones [33,34,99]. The complex Iberian orography may have allowed partial differentiation of lineages in allopatry but subsequent secondary contacts of differentiated genomes from close locations [34]. That may have been the framework for *Linaria* and many other southern European plant groups (Table 8). Clearly, the investigation of hybridization in Mediterranean plant groups is vital for the accurate inference of species trees, as well as to understand the role of hybridization in the generation of new genetic combinations and morphological differentiation. However, we have shown in this example that existing tools, although limited, can nonetheless provide valuable insights in these areas.

Incomplete Lineage Sorting as a Significant Process in Mediterranean Plants

Several studies have claimed incomplete lineage sorting as a major cause of gene tree incongruence and non-monophyly in Mediterranean plants (Table 8). Failure of gene lineages to coalesce occurs when the time between speciation events is very short and/or when the effective population size of the ancestral populations is very large [2]. We detected incomplete lineage sorting in all independent loci analyzed for *Linaria*. In this genus, population size estimates obtained by using three *Linaria* species (*L. glacialis*, *L. elegans*, *L. simplex*) suggested that ancestral populations may have not been extremely large (see [107] for comparison). Conversely, extremely rapid divergence of ancestral populations seems more likely. *Linaria* has diversified since the late Miocene-early Pliocene (3.57–12.14 Ma) (crown node of the genus, Table 5) to recent times in the late Quaternary (Table 5, Fig. 4). During its evolutionary history, this Mediterranean group may have experienced drastic climatic events such as the Messinian Salinity Crisis (5.96 Ma) [108], the catastrophic flood that caused the refilling of the Mediterranean Sea (5.33 Ma) [109], the progressive establishment of the Mediterranean rhythm with dry summers (3.2 Ma) and the Quaternary type oscillations with glacial and interglacial stages (2.3 Ma) [39]. These extreme climatic changes coupled with the irregular mountain ranges of the Mediterranean basin might have promoted rapid diversification driven by isolation in reduced areas causing rapid allopatric speciation. The secondary contacts occurring during the climatic oscillations seem to have promoted historical hybridization between closely related *Linaria* species, but also the high number of species in the Mediterranean (104 spp.) [45] and its recent origin suggest that this group is likely to have undergone rapid diversification. Additional analyses not performed here are proposed to confirm rapid speciation as the cause for incomplete lineage sorting in *Linaria*.

The basis underlying phylogenetic incongruence may vary depending on the plant group under study, but the flora of the Mediterranean is formed, in part, by many genera that similarly display numerous species generated in short periods of time that also may have suffered secondary contacts in short term cycles (20.000–100.000 yr.). In these groups incomplete lineage sorting and hybridization appear to be the rule rather than the exception.

Supporting Information

Figure S1 Total evidence analysis. The 50% majority-rule consensus tree obtained in the Bayesian analysis of the concatenated ITS, AGT1 and cpDNA datasets. Numbers above branches are Bayesian posterior probabilities. Colors represent the systematic nomenclature for *Supinae* as suggested in this paper (see Fig. 4). Species with intermediate key traits are represented in grey.
(TIF)

Table S1 List of taxa included with localities, collector's numbers and Genbank accession numbers.
(DOCX)

Table S2 Effective population size estimates (N_e) used in the coalescent simulations.
(DOCX)

Table S3 Assignment of genes to label 1 (L1) or label 2 (L2) in the multilabelled species tree analysis (Fig. 5).
(DOCX)

Methods S1 Supplemental methods.
(DOCX)

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Author Contributions

Conceived and designed the experiments: JLB-P PV BEP. Performed the experiments: JLB-P BEP. Analyzed the data: JLB-P BEP. Contributed reagents/materials/analysis tools: JLB-P PV BEP. Wrote the paper: JLB-P PV BEP.

References

1. Doyle JJ (1992) Gene trees and species trees: Molecular systematics as one-character taxonomy. Systematic Botany 17: 144–163.
2. Maddison WP (1997) Gene Trees in Species Trees. Systematic Biology 46: 523–536.
3. Pamilo P, Nei M (1988) Relationships between gene trees and species trees. Molecular biology and evolution 5: 568–583.
4. Rosenberg NA, Nordborg M (2002) Genealogical trees, coalescent theory and the analysis of genetic polymorphisms. Nature Review Genetics 3: 380–390.
5. Wendel JF, Doyle JJ (1998) Phylogenetic incongruence: Window into genome history and molecular evolution. Pages 265–296. In: Soltis DE, Soltis PS, Doyle JJ, editors. Molecular systematics of plants II: DNA Sequencing. Norwell: Kluwer Academic Publishers.
6. Maureira-Butler IJ, Pfeil BE, Muangprom A, Osborn TC, Doyle JJ (2008) The reticulate history of *Medicago* (Fabaceae). Systematic Biology 57: 466–482.
7. Buckley TR, Cordeiro M, Marshall DC, Simon C (2006) Differentiating between hypotheses of lineage sorting and introgression in New Zealand alpine cicadas (Maoricicada Dugdale). Systematic Biology 55: 411–425.
8. Peters JL, Zhuravlev Y, Fefelov I, Logie A, Omland KE (2007) Nuclear loci and coalescent methods support ancient hybridization as cause of mitochondrial paraphyly between gadwall and falcated duck (*Anas* spp.). Evolution 61: 1992–2006.
9. van der Niet T, Linder HP (2008) Dealing with incongruence in the quest for the species tree: A case study from the orchid genus Satyrium. Molecular Phylogenetics and Evolution 47: 154–174.
10. Joly S, McLenachan PA, Lockhart PJ (2009) A statistical approach for distinguishing hybridization and incomplete lineage sorting. American Naturalist 174: E54–E70.
11. Frajman B, Eggens F, Oxelman B (2009) Hybrid origins and homoploid reticulate evolution within *Heliosperma* (Sileneae, Caryophyllaceae)-A multigene phylogenetic approach with relative dating. Systematic Biology 58: 328–345.
12. Stone RD, Andreasen K (2010) The Afro-Madagascan genus Warneckea (Melastomataceae): Molecular systematics and revised infrageneric classification. Taxon 59: 83–92.
13. Wong SY, Boyce PC, Sofiman bin Othman A, Pin LC (2010) Molecular phylogeny of tribe Schismatoglottideae (Araceae) based on two plastid markers and recognition of a new tribe, Philonotieae, from the neotropics. Taxon 59: 117–124.
14. Link P, rez MA, Watson LE, Hickey RJ (2011) Redefinition of Adiantopsis Fee (Pteridaceae): Systematics, diversification, and biogeography. Taxon 60: 1255–1268.
15. Martínez-Azorín M, Crespo MB, Juan A, Fay MF (2011) Molecular phylogenetics of subfamily Ornithogaloideae (Hyacinthaceae) based on nuclear and plastid DNA regions, including a new taxonomic arrangement. Annals of Botany 107: 1–37.
16. Liu ZW, Zhou J, Liu ED, Peng H (2010) A molecular phylogeny and a new classification of *Pyrola* (Pyroleae, Ericaceae). Taxon 59: 1690–1700.
17. Emadzade K, Lehnebach C, Lockhart P, Hörandl E (2010) A molecular phylogeny, morphology and classification of genera of Ranunculeae (Ranunculaceae). Taxon 59: 809–828.
18. Roxanne Steele P, Friar LM, Gilbert LE, Jansen RK (2010) Molecular systematics of the neotropical genus *Psiguria* (Cucurbitaceae): Implications for phylogeny and species identification. American Journal of Botany 97: 156–173.
19. Kubatko LS, Degnan JH (2007) Inconsistency of phylogenetic estimates from concatenated data under coalescence. Systematic Biology 56: 17–24.
20. Heled J, Drummond AJ (2010) Bayesian Inference of Species Trees from Multilocus Data. Molecular Biology and Evolution 27: 570–580.
21. Yu Y, Cuong T, Degnan JH, Nakhleh L (2011) Coalescent Histories on Phylogenetic Networks and Detection of Hybridization Despite Incomplete Lineage Sorting. Systematic Biology 60: 138–149.
22. Bloomquist EW, Suchard MA (2010) Unifying Vertical and Nonvertical Evolution: A Stochastic ARG-based Framework. Systematic Biology 59: 27–41.
23. Holland B, Benthin S, Lockhart P, Moulton V, Huber K (2008) Using supernetworks to distinguish hybridization from lineage-sorting. BMC Evolutionary Biology 8: 202.
24. Ané C, Larget B, Baum DA, Smith SD, Rokas A (2007) Bayesian estimation of concordance among gene trees. Molecular Biology and Evolution 24: 412–426.
25. Edwards SV, Liu L, Pearl DK (2007) High-resolution species trees without concatenation. Proceedings of the National Academy of Sciences 104: 5936–5941.
26. Carstens BC, Knowles LL (2007) Estimating species phylogeny from gene-tree probabilities despite incomplete lineage sorting: An example from *Melanoplus* grasshoppers. Systematic Biology 56: 400–411.
27. Liu L, Pearl DK, Brumfield RT, Edwards SV (2008) Estimating species trees using multiple-allele DNA sequence data. Evolution 62: 2080–2091.
28. Liu L, Yu L, Pearl DK, Edwards SV (2009) Estimating Species Phylogenies Using Coalescence Times among Sequences. Systematic Biology 58: 468–477.
29. Fan HH, Kubatko LS (2011) Estimating species trees using approximate Bayesian computation. Molecular Phylogenetics and Evolution 59: 354–363.
30. Liu L, Yu L, Kubatko L, Pearl DK, Edwards SV (2009) Coalescent methods for estimating phylogenetic trees. Molecular Phylogenetics and Evolution 53: 320–328.
31. Mallet J (2007) Hybrid speciation. Nature 446: 279–283.
32. Hewitt GM (2004) Genetic consequences of climatic oscillations in the Quaternary. Philosophical Transactions of the Royal Society B: Biological Sciences 359: 183–195.
33. Gómez A, Lunt D (2006) Refugia within refugia: patterns of phylogeographic concordance in the Iberian Peninsula. In: Weiss S, Ferrand N, editors. Phylogeography of Southern European Refugia. The Netherlands: Springer. 155–188.
34. Feliner GN (2011) Southern European glacial refugia: A tale of tales. Taxon 60: 365–372.
35. Belfiore NM, Liu L, Moritz C (2008) Multilocus phylogenetics of a rapid radiation in the genus Thomomys (Rodentia: Geomyidae). Systematic Biology 57: 294–310.
36. Fiz-Palacios O, Vargas P, Vila R, Papadopulos AST, Aldasoro JJ (2010) The uneven phylogeny and biogeography of *Erodium* (Geraniaceae): radiations in the Mediterranean and recent recurrent intercontinental colonization. Annals of Botany 106: 871–884.
37. Valente LM, Savolainen V, Vargas P (2010) Unparalleled rates of species diversification in Europe. Proceedings of the Royal Society B: Biological Sciences 277: 1489–1496.
38. Guzmán B, Lledó MD, Vargas P (2009) Adaptive Radiation in Mediterranean *Cistus* (Cistaceae). Plos One 4: e6362.
39. Suc JP (1984) Origin and evolution of the Mediterranean vegetation and climate in Europe. Nature 307: 429–432.
40. Fernández-Mazuecos M, Vargas P (2011) Historical Isolation versus Recent Long-Distance Connections between Europe and Africa in Bifid Toadflaxes (*Linaria* sect. *Versicolores*). Plos One 6.
41. Hsu KJ, Montadert L, Bernoulli D, Cita MB, Erickson A, et al. (1977) History of the Mediterranean salinity crisis. Nature 267: 399–403.
42. Vargas P, Rossello JA, Oyama R, Guemes J (2004) Molecular evidence for naturalness of genera in the tribe Antirrhineae (Scrophulariaceae) and three independent evolutionary lineages from the New World and the Old. Plant Systematics and Evolution 249: 151–172.
43. Rothmaler W (1943) Zur Gliederung der Antirrhineae. Feddes Repertorium Specierum Novarum Regni Vegetabilis 52: 16–39.
44. Sáez L, Bernal M (2009) Linaria Mill. In: Castroviejo S, Herrero A, Benedí C, Rico E, Güemes J, editors. Flora Ibérica 13. Madrid: CSIC. 232–324.
45. Sutton DA (1988) A revision of the tribe Antirrhineae. London. UK: Oxford University Press.
46. Valdés B (1970) Revisión de las especies europeas de *Linaria* con semillas aladas. Sevilla. Spain: Anales de la Universidad Hispalense. 1–288 p.
47. Wettstein (1895) Scrophulariaceae. In: Prantl E, editor. Die Natürligen Pflanzenfamilien. 39–107.
48. Bentham G (1846) Scrophulariaceae In: Candolle A-L-P-Pde, editor. Prodromus Sistematis Universalis Regni Vegetabilis. 266–288.
49. Viano J (1978) Les linaires à graines aptères du bassin méditerranéen occidental. 1. *Linaria* sect. *Versicolores*. Candollea 33: 33–88.
50. Viano J (1978) Les linaires à graines aptères du bassin méditerranéen occidental. 2. *Linaria* sect. *Elegantes*, *Bipunctatae*, *Diffusae*, *Speciosae*, *Repentes*. Candollea 33: 209–267.
51. Chavannes E (1833) Monographie des Antirrhinées: Paris et Lausanne.
52. Valdés B (1970) Taxonomía experimental del género *Linaria* III. Cariología de algunas especies de *Linaria*, *Cymbalaria* y *Chaenorrhinum*. Boletín de la Real Sociedad Española de Historia Natural (Biología) 67: 243–256.
53. Valdés B (1970) Taxonomía experimental del género *Linaria* V. Hibridacion interespecifica. Acta Phytotaxonomica Barcinonensia 4.
54. Viano J (1978) Croisements experimentaux interspecifiques au sein du genre *Linaria*. Caryologia 31(4): 383–425.
55. Rouy G (1909) des Tribus et des Genres de la famille des Scrofulariacées. In: Deyrolle LFdÉ, editor. Flore de France ou description des plantes qui croissent spontanement en France, en Corse et en Alsace-Lorraine. Paris.
56. Druce GC (1925) L. repens x supina nov. hybr. Botanical Society and Exchange Club of the British Isles 7: 998.
57. Fournier P (1946) Les Quatre Flores de la France. Paris: Corse Comprise.
58. Seehausen O (2004) Hybridization and adaptive radiation. Trends in Ecology and Evolution 19: 198–207.
59. Liepman AH, Olsen LJ (2001) Peroxisomal alanine : glyoxylate aminotransferase (AGT1) is a photorespiratory enzyme with multiple substrates in Arabidopsis thaliana. The Plant Journal 25: 487–498.
60. White TJ, Bruns T., Lee S. and Taylor J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In "PCR Protocols. A Guide to Methods and Applications"; M.A. Innis DHG, J.J. Sninsky and T. J. White, Eds., editor. San Diego. USA: Academic Press.
61. Hamilton MB (1999) Four primer pairs for the amplification of chloroplast intergenic regions with intraspecific variation. Molecular Ecology 8: 521–523.
62. Shaw J, Lickey EB, Schilling EE, Small RL (2007) Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: The Tortoise and the hare III. American Journal of Botany 94: 275–288.

63. Sáez L, Crespo MB (2005) A taxonomic revision of the Linaria verticillata group (Antirrhineae, Scrophulariaceae). Botanical Journal of the Linnean Society 148: 229–244.
64. del Campo P, del Amo M (1855) Especies de plantas nuevas descubiertas por D. Pedro del Campo y descritas por D. Mariano del Amo. Revista de los Progresos de las Ciencias Exactas, Físicas y Naturales 5: 55–58.
65. Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. Molecular Ecology Resources 10: 564–567.
66. Excoffier L, Laval G, Balding D (2003) Gametic phase stimation over large genomic regions using an adaptive window approach. Human Genomics 1:7–9.
67. Scheen A-C, Pfeil BE, Petri A, Heidari N, Nylinder S, et al. (2011) Use of allele-specific sequencing primers is an efficient alternative to PCR subcloning of low-copy nuclear genes. Molecular Ecology Resources 12: 128–135.
68. Heath L, Van Der Walt E, Varsani A, Martin DP (2006) Recombination patterns in aphthoviruses mirror those found in other picornaviruses. Journal of Virology 80: 11827–11832.
69. Martin D, Rybicki E (2000) RDP: Detection of recombination amongst aligned sequences. Bioinformatics 16: 562–563.
70. Padidam M, Sawyer S, Fauquet CM (1999) Possible emergence of new geminiviruses by frequent recombination. Virology 265: 218–225.
71. Smith JM (1992) Analyzing the mosaic structure of genes. Journal of Molecular Evolution 34: 126–129.
72. Martin DP, Posada D, Crandall KA, Williamson C (2005) A modified bootscan algorithm for automated identification of recombinant sequences and recombination breakpoints. AIDS Research and Human Retroviruses 21: 98–102.
73. Gibbs MJ, Armstrong JS, Gibbs AJ (2000) Sister-Scanning: a Monte Carlo procedure for assessing signals in recombinant sequences. Bioinformatics 16: 573–582.
74. Boni MF, Posada D, Feldman MW (2007) An exact nonparametric method for inferring mosaic structure in sequence triplets. Genetics 176: 1035–1047.
75. Posada D, Crandall KA (2001) Evaluation of methods for detecting recombination from DNA sequences: Computer simulations. Proceedings of the National Academy of Sciences of the United States of America 98: 13757–13762.
76. Ronquist F, Huelsenbeck JP (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574.
77. Katoh K, Toh H (2008) Recent developments in the MAFFT multiple sequence alignment program. Briefings in Bioinformatics 9: 286–298.
78. Posada D (2008) jModelTest: Phylogenetic model averaging. Molecular biology and evolution 25: 1253–1256.
79. Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, et al. (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Systematic Biology 59: 307–321.
80. Bell CD, Soltis DE, Soltis PS (2010) The age and diversification of the angiosperms re-revisited. American Journal of Botany 97: 1296–1303.
81. Sanderson MJ (2003) r8s: Inferring absolute rates of molecular evolution and divergence times in the absence of a molecular clock. Bioinformatics 19: 301–302.
82. Heled J, Drummond A (2008) Bayesian inference of population size history from multiple loci. BMC Evolutionary Biology 8: 289.
83. Li H, Durbin R (2011) Inference of human population history from individual whole-genome sequences. Nature 475: 493–496.
84. Shimodaira H, Hasegawa M (1999) Multiple comparisons of log-likelihoods with applications to phylogenetic inference. Molecular Biology and Evolution 16: 1114–1116.
85. Kass RE, Raftery AE (1995) Bayes factors. Journal of the American Statistical Association 90: 773–795.
86. Suchard MA, Weiss RE, Sinsheimer JS (2001) Bayesian selection of continuous-time Markov chain evolutionary models. Molecular Biology and Evolution 18: 1001–1013.
87. Shimodaira H, Hasegawa M (2002) CONSEL: For assessing the confidence of phylogenetic tree selection. Bioinformatics 17: 1246–1247.
88. Rambaut A, Drummond AJ (2007) Tracer v1.4, Available from BEAST Software website: http://beast.bio.ed.ac.uk/Tracer.Accessed November 2011.
89. Drummond A, Rambaut A (2007) BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evolutionary Biology 7: 214.
90. Gernhard T (2008) The conditioned reconstructed process. Journal of Theoretical Biology 253: 769–778.
91. Kay KM, Whittall JB, Hodges SA (2006) A survey of nuclear ribosomal internal transcribed spacer substitution rates across angiosperms: an approximate molecular clock with life history effects. BMC Evolutionary Biology 6.
92. Wolfe KH, Li WH, Sharp PM (1987) Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast, and nuclear DNAs. Proceedings of the National Academy of Sciences 84: 9054–9058.
93. Senchina DS, Alvarez I, Cronn RC, Liu B, Rong JK, et al. (2003) Rate variation among nuclear genes and the age of polyploidy in *Gossypium*. Molecular Biology and Evolution 20: 633–643.
94. Pfeil BE, Schlueter JA, Shoemaker RC, Doyle JJ (2005) Placing paleopolyploidy in relation to taxon divergence: A phylogenetic analysis in legumes using 39 gene families. Systematic Biology 54: 441–454.
95. Pirie MD, Humphreys AM, Barker NP, Linder HP (2009) Reticulation, Data Combination, and Inferring Evolutionary History: An Example from Danthonioideae (Poaceae). Systematic Biology 58: 612–628.
96. Poke FS, Martin DP, Steane DA, Vaillancourt RE, Reid JB (2006) The impact of intragenic recombination on phylogenetic reconstruction at the sectional level in Eucalyptus when using a single copy nuclear gene (cinnamoyl CoA reductase). Molecular Phylogenetics and Evolution 39: 160–170.
97. Degnan JH, Rosenberg NA (2009) Gene tree discordance, phylogenetic inference and the multispecies coalescent. Trends in Ecology and Evolution 24: 332–340.
98. Postigo-Mijarra JM, Morla C, Barrón E, Morales-Molino C, García S (2010) Patterns of extinction and persistence of Arctotertiary flora in Iberia during the Quaternary. Review of Palaeobotany and Palynology 162: 416–426.
99. Hewitt G (2011) Quaternary phylogeography: the roots of hybrid zones. Genetica 139: 617–638.
100. Martín-Bravo S, Valcárcel V, Vargas P, Luceño M (2010) Geographical speciation related to Pleistocene range shifts in the western mediterranean mountains (*Reseda* sect. *Glaucoreseda*, Resedaceae). Taxon 59: 466–482.
101. Vargas P, Carrió E, Guzmán B, Amat E, Güemes J (2009) A geographical pattern of *Antirrhinum* (Scrophulariaceae) speciation since the Pliocene based on plastid and nuclear DNA polymorphism. Journal of Biogeography
102. Wilson Y, Hudson A (2011) The evolutionary history of *Antirrhinum* suggests that ancestral phenotype combinations survived repeated hybridizations. Plant Journal 66: 1032–1043.
103. Abbott RJ, Hegarty MJ, Hiscock SJ, Brennan AC (2010) Homoploid hybrid speciation in action. Taxon 59: 1375–1386.
104. Buerkle CA, Morris RJ, Asmussen MA, Rieseberg LH (2000) The likelihood of homoploid hybrid speciation. Heredity 84: 441–451.
105. Hewitt GM (1999) Post-glacial re-colonization of European biota. Biological Journal of the Linnean Society 68: 87–112.
106. Hewitt G (2000) The genetic legacy of the quaternary ice ages. Nature 405: 907–913.
107. Gossmann TI, Song B-H, Windsor AJ, Mitchell-Olds T, Dixon CJ, et al. (2010) Genome Wide Analyses Reveal Little Evidence for Adaptive Evolution in Many Plant Species. Molecular Biology and Evolution 27: 1822–1832.
108. Krijgsman W, Hilgen FJ, Raffi I, Sierro FJ, Wilson DS (1999) Chronology, causes and progression of the Messinian salinity crisis. Nature 400: 652–655.
109. Garcia-Castellanos D, Estrada F, Jiménez-Munt I, Gorini C, Fernàndez M, et al. (2009) Catastrophic flood of the Mediterranean after the Messinian salinity crisis. Nature 462: 778–781.
110. Barres L, Vilatersana R, Molero J, Susanna A, Galbany-Casals M (2011) Molecular phylogeny of *Euphorbia* subg. *Esula* sect. *Aphyllis* (Euphorbiaceae) inferred from nrDNA and cpDNA markers with biogeographic insights. Taxon 60: 705–720.
111. Presti RML, Oppolzer S, Oberprieler C (2010) A molecular phylogeny and a revised classification of the mediterranean genus *Anthemis* s.l. (Compositae, Anthemideae) based on three molecular markers and micromorphological characters. Taxon 59: 1441–1456.
112. Mansion G, Zeltner L, Bretagnolle F (2005) Phylogenetic patterns and polyploid evolution within the Mediterranean genus *Centaurium* (Gentianaceae - Chironieae). Taxon 54: 931–950.
113. Vilatersana R, Garcia-Jacas N, Garnatje T, Molero J, Sonnante G, et al. (2010) Molecular phylogeny of the genus *Ptilostemon* (Compositae: Cardueae) and its relationships with *Cynara* and *Lamyropsis*. Systematic Botany 35: 907–917.
114. Jakob SS, Blattner FR (2006) A chloroplast genealogy of *Hordeum* (Poaceae): Long-term persisting haplotypes, incomplete lineage sorting, regional extinction, and the consequences for phylogenetic inference. Molecular Biology and Evolution 23: 1602–1612.
115. Meerow AW, Francisco-Ortega J, Kuhn DN, Schnell RJ (2006) Phylogenetic relationships and biogeography within the Eurasian clade of Amaryllidaceae based on plastid ndhF and nrDNA ITS sequences: Lineage sorting in a reticulate area? Systematic Botany 31: 42–60.
116. Guo YP, Ehrendorfer F, Samuel R (2004) Phylogeny and systematics of *Achillea* (Asteraceae-Anthemideae) inferred from nrITS and plastid trnL-F DNA sequences. Taxon 53: 657–672.
117. Comes HP, Abbott RJ (2001) Molecular phylogeography, reticulation, and lineage sorting in mediterranean *Senecio* sect. *Senecio* (Asteraceae). Evolution 55: 1943–1962.
118. Valcárcel V, Vargas P, Feliner GN (2006) Phylogenetic and phylogeographic analysis of the western Mediterranean *Arenaria* section *Plinthine* (Caryophyllaceae) based on nuclear, plastid, and morphological markers. Taxon 55: 297–312.
119. Albaladejo RG, Aguilar JF, Aparicio A, Feliner GN (2005) Contrasting nuclear-plastidial phylogenetic patterns in the recently diverged Iberian *Phlomis crinita* and *P. lychnitis* lineages (Lamiaceae). Taxon 54: 987–998.

Supporting Information from Blanco-Pastor *et al.* 2012, "Coalescent Simulations Reveal Hybridization and Incomplete Linage Sorting in Mediterranean *Linaria*"

Plos ONE 7(6): e39089

Figure S1

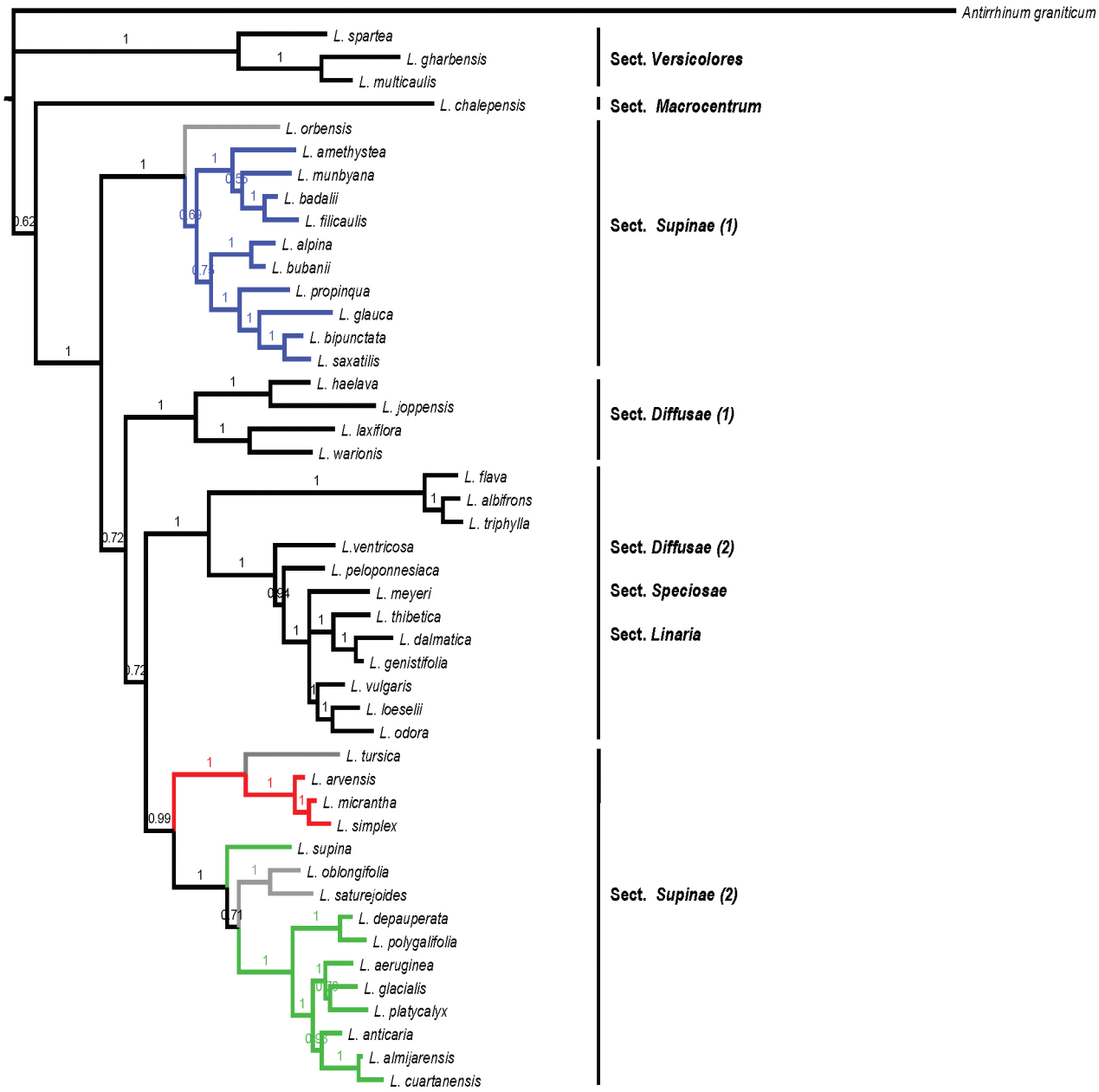
Antirrhinum graniticum
L. spartea
L. gharbensis
L. multicaulis
L. chalepensis
L. orbensis
L. amethystea
L. munbyana
L. badalii
L. filicaulis
L. alpina
L. bubanii
L. propinqua
L. glauca
L. bipunctata
L. saxatilis
L. haelava
L. joppensis
L. laxiflora
L. warionis
L. flava
L. albifrons
L. triphylla
L.ventricosa
L. peloponnesiaca
L. meyeri
L. thibetica
L. dalmatica
L. genistifolia
L. vulgaris
L. loeselii
L. odora
L. tursica
L. arvensis
L. micrantha
L. simplex
L. supina
L. oblongifolia
L. saturejoides
L. depauperata
L. polygalifolia
L. aeruginea
L. glacialis
L. platycalyx
L. anticaria
L. almijarensis
L. cuartanensis
Sect. Versicolores
Sect. Macrocentrum
Sect. Supinae (1)
Sect. Diffusae (1)
Sect. Diffusae (2)
Sect. Speciosae
Sect. Linaria
Sect. Supinae (2)

Table S1. List of taxa included in the present paper with localities, collector's numbers and Genbank accession numbers.

Taxon	Locality	Collector	Collection number (herbarium)	ITS	*trnS-trnG*	*rpl32-trnL*^{UAG}	*Agt1*
Sect. *Supinae*							
L. aeruginea (Gouan) Cav. ssp. *aeruginea*	Spain: Granada. Sierra Nevada. Pradollano	J.L. Blanco Pastor	51JB09	JQ814486	JN663616	JN663397	JQ814558
L. aeruginea ssp. *nevadensis* (Boiss.) D.A. Sutton	Spain: Granada. Sierra Nevada. Pico del Veleta	J.L. Blanco Pastor	44JB09	JQ814487	JN663620	JN663401	JQ814559
L. almijarensis Campo & Amo	Spain: Córdoba. Cabra.	J.L. Blanco Pastor	36JB10	JQ814492	JN663632	JN663412	JQ814564
L. alpina (L.) Mill.	Spain: Huesca. Formigal	S. Martín Bravo	571SMB05	JQ814489	JN663622	JN663510	JQ814561
L. amethystea (Vent.) Hoffmanns. & Link *ssp. amethystea*	Spain: Ciudad Real. Ciudad Real	R. García Río	712742 (MA)	JQ814490	JN663624	JN663405	JQ814562
L. anticaria Boiss. & Reut.	Spain: Málaga. El Torcal de Antequera	J.L. Blanco Pastor	33JB09	JQ814491	JN663628	JN663408	JQ814563
L. arvensis (L.) Desf. (1)	France: Córse. Col de Bigorno.	J. Lambinon	2009.12.169 (RNG)	JQ814493	JN663635	JN663415	JQ814565
L. arvensis (L.) Desf. (2)	Spain: Almería. Uleila del Campo	S.L. Jury & R.N. Carter	2009.12.165 (RNG)	JQ814494	JN663636	JN663416	JQ814566
L. badalii Loscos	Spain: León. Riaño	M.F. Gardner & S.G. Gardner	2009.12.155 (RNG)	JQ814495	JN663641	JN663421	JQ814567
L. bipunctata (L.) Chaz. ssp. *bipunctata*	Spain: Soria. Quintana Redonda	A. Segura	2009.12.7 (RNG)	JQ814496	JN663643	JN663423	JQ814568
L. bubanii Font Quer	Spain: Huesca. El Pueyo de Araguas	M. Carrasco & al.	609430 (MA)	JQ814537 JQ814538	JN663644	JN663424	JQ814569
L. cuartanensis (Degen & Hervier) Fern. Casas ex. Blanco-Pastor	Spain: Albacete. Yeste	P. F. Cannon et al.	2009.12.35 (RNG)	JQ814510	JN663667	JN663444	JQ814583
L. depauperata Leresche ex Lange ssp. *depauperata*	Spain: Alicante. Alcoi	L. Serra	2009.12.157 (RNG)	JQ814499	JN663651	JN663430	JQ814572
L. filicaulis Boiss. ex Leresche & Levier	Spain: León. Pico Tres Provincias	C.M. Romero Rodríguez	769084 (MA)	JQ814500	JN663655	JN663434	JQ814573
L. glacialis Boiss. (1)	Spain: Granada. Sierra Nevada. Corral del Veleta	J.L. Blanco Pastor	43JB09	JQ814504	JN663659	JN663437	JQ814577
L. glacialis Boiss. (2)	Spain: Granada. Sierra Nevada. El Caballo.	J.L. Blanco Pastor	70JB09	JQ814505	JN663660	JN663438	JQ814578
L. glauca ssp. olcadium Valdés & D.A. Webb	Spain: Albacete. Balazote	Rivas Goday	2009.12.151 (RNG)	JQ814506	JN663663	JN663441	JQ814579
L. micrantha (Cav.) Hoffmanns. & Link.	Spain: Huelva. Marismas del Odiel	J.L. Blanco Pastor	22JB09	JQ814513	JN663669	JN663446	JQ814585
L. munbyana Boiss. & Reut.	Spain: Huelva. Marismas del Odiel	J.L. Blanco Pastor	21JB09	JQ814515	JN663670	JN663447	JQ814587
L. oblongifolia (Boiss.) Boiss. & Reut. *ssp. oblongifolia*	Spain: Málaga. El Torcal de Antequera	J.L. Blanco Pastor	34JB09	JQ814516	JN663672	JN663449	JQ814588

L. orbensis Carretero & Boira.	Spain: Alicante. Sagra	J.L. Blanco Pastor	4JB10	JQ814518	JN663676	JN663453	JQ814590
L. platycalyx Boiss.	Spain: Cádiz. Zahara de la Sierra	S. Martín Bravo	5SMB08	JQ814520	JN663677	JN663454	JQ814592
L. polygalifolia ssp. lamarckii (Rouy) D.A. Sutton. (1)	Portugal: Algarve. Monte Gordo	J.L. Blanco Pastor	33JB10	JQ814522	JN663679	JN663456	JQ814594
L. polygalifolia ssp. lamarckii (Rouy) D.A. Sutton. (2)	Spain: Huelva. Isla Canela.	J.L. Blanco Pastor	19JB09	JQ814521	JQ814550	JQ814617	JQ814593
L. polygalifolia Hoffmanns. & Link *ssp. polygalifolia*	Portugal: Estremadura. Guincho	H.J.M. Bowen	2009.12.108 (RNG)	JQ814523	JN663680	JN663457	JQ814479 JQ814483
L. propinqua Boiss. & Reut.	Spain: Bilbao. Zeanurri	J.A. Alejandre	468162 (MA)	JQ814524	JN663682	JN663459	JQ814595
L. saturejoides Boiss. ssp. *saturejoides*	Spain: Málaga. Sierra Tejeda. Canillas de Aceituno	J.L. Blanco Pastor	36JB09	JQ814525	JN663685	JN663462	JQ814596
L. saxatilis (L.) Chaz.	Spain: Ávila. Hoyos del Espino	P. Vargas	94PV09	JQ814526	JN663687	JN663464	JQ814597
L. simplex Willd. ex Desf. (1)	Greece: Ararchova	P. Vargas	79PV08	JQ814528	JN663697	JN663474	JQ814599
L. simplex Willd. ex Desf. (2)	Spain: Granada. Orjiva	R.N. Carter	2009.12.162 (RNG)	JQ814527	JN663699	JN663476	JQ814598
L. supina (L.) Chaz. *ssp. supina*	France: Gorges de l'Hérault	J. Lambinon	2009.12.131 (RNG)	JQ814530	JN663704	JN663481	JQ814601
L. tursica Valdés & Cabezudo.	Spain: Huelva. Coto de Doñana	J.L. Blanco Pastor	18JB09	JQ814533	JN663715	JN663492	JQ814603
Other sections							
L. albifrons (Sibth. & Sm.) Steudel (Sect. Diffusae)	Israel. Negev. Beer Sheva	A. Danin, S.G. Knees et al.	2010/12/07 (RNG)	JQ814488	JQ814539	JQ814606	JQ814560
L. chalepensis (L.) Mill. (Sect. Macrocentrum)	Cyprus: Larnaca, Cape Kiti	Iter Mediterraneum IV	495681(MA)	JQ814497	JN663647	JF694128	JQ814570
L. dalmatica (L.) Miller (Sect. Speciossae)	Bulgaria: Central Rhodopes	C. Navarro & al.	726987 (MA)	JQ814498	JQ814540	JQ814607	JQ814571
L. flava (Poiret) Desf. (Sect. Diffusae)	Italy: Corsica. Camp dell'Oro. Ajaccio	C. Bukanell & L. Ollum	00419551 (E)	JQ814501	JQ814541	JQ814608	JQ814574
L. genistifolia (L.) Miller (Sect. Speciosae)	Turkey: Hadim-Bezkir	JJ. Aldasoro & M.L. Alarcón	A9751	JQ814502	JQ814542	JQ814609	JQ814575
L. gharbensis Batt. & Pit. (Sect. Versicolores)	Spain: Huelva. Gibraleón	M. Fernández-Mazuecos	7MF09	JQ814503	JN663658	JF694139	JQ814576
L. haelava (Forskål) F.G. Dietr. (Sect. Diffusae)	Israel: Horbat Medin	D. Heller & I. Shammash	532177 (MA)	JQ814507	JQ814543	JQ814610	JQ814580
L. joppensis Bornm. (Sect. Diffusae)	Israel: Philistean plain. Ashkeleton	A. Danin, S.G. Knees et al.	2010/12/11 (RNG)	JQ814508	JQ814544	JQ814611	JQ814581
L. laxiflora Desf. (Sect. Diffusae)	Tunisia: Jerid. Cedada. Sidi Ben Arbessidi Bouhlel	C. Aedo & al.	795183 (MA)	JQ814509	JQ814545	JQ814612	JQ814582
L. loeselii Schweiger (Sect. Linaria)	Lithuania. Apskritis ol Klaipeda. Curonian Spit.	E. Glazkova & A. Quintanar	791644 (MA)	JQ814511	JQ814546	JQ814613	JQ814584
L.meyeri Kuprian (Sect. Linaria)	Georgia: Mtskhete Mtianeti. Gran Caucaso	L. Muñoz & al.	764400 (MA)	JQ814512	JQ814547	JQ814614	JQ814476

							JQ814480
L. multicaulis (L.) Mill. (Sect. Versicolores)	Morocco: Azrou	M. Fernández-Mazuecos	15MF08	JQ814514	JN663719	JF694155	JQ814586
L. odora (Bieb.) Fisher (Sect. Linaria)	Russia. Voilgograd. Frolavo. Prov. Gulajerka	A.K. Skvortsov	00419545 (E)	JQ814517	JQ814548	JQ814615	JQ814589
L. peloponnesiaca Boiss. & Heldr. (Sect. Speciosae)	Greece: Mt. Olympus	P. Vargas	778352 (MA)	JQ814519	JQ814549	JQ814616	JQ814591
L. spartea (L.) Chaz. (Sect. Versicolores)	Spain: Cáceres. Monfragüe	M. Fernández-Mazuecos	4MF08	JQ814529	JN663701	JN663478	JQ814600
L. thibetica Franchet (Sect. Linaria)	China: Sichuan prov. Siangcheng Xian. between Xiangcheng and Daxue	D.E. Boufford & al.	00292244 (E)	JQ814531	JQ814551	JQ814618	JQ814602
L. triphylla (L.) Miller (Sect. Diffusae)	Tunisia: El Vef. between Sidi Merzong and Aïn Linghassel	J. Calvo & al.	797461 (MA)	JQ814532	JQ814552	JQ814619	JQ814478 JQ814482
L. ventricosa Cosson & Bal. (Sect. Speciosae)	Morocco: Meknès-Tafilalet	T. Buira, J. Calvo & S. Hartson	807960 (MA)	JQ814534	JQ814553	JQ814620	JQ814604
L. vulgaris Miller (Sect. Linaria)	France: Chamonix	B. Estébanez	s.n.	JQ814535	JQ814554	JQ814621	JQ814477 JQ814481
L.warionis Pomel (Sect. Diffusae)	Morocco: Beni Tajjita, route vers Talsinat	D.Podlech	589733 (MA)	JQ814536	JQ814555	JQ814622	JQ814605
Outgroup							
Antirrhinum graniticum Rothm.	Spain: Cáceres. Trujillo	P. Vargas	213PV06	JQ814484	JN663609	JF694120	JQ814556
Chaenorhinum.macropodum (Boiss. & Reut.) Lange	Spain: Málaga. Cómpeta	M. Fernández-Mazuecos	7E3MF08	JQ814485	JN663610	JF694119	JQ814557

Table S2. Effective population size estimates (N_e) used in the coalescent simulations.

	Simulations from ITS and AGT1trees	Simulations from cpDNA trees
L. elegans	320 000	160 000
L. glacialis	190 000	95 000
L. simplex	680 000	340 000

Table S3. Assignment of genes to label 1 (L1) or label 2 (L2) in the multilabelled species tree analysis (Fig. 5).

Presumed hybrid	**ITS**	**AGT1**	**cpDNA**
L. glauca ssp. olcadium	L1	L2	L1
L. orbensis	L1	L1	L2
L. amethystea ssp. *amethystea*	L1	L2	L1
L. cuartanensis	L1	L2	L1
L. tursica	L1	L1	L2
L. oblongifolia ssp. *oblongifolia*	L1	L2	L1
L. alpina	L1	L1	L2
L. filicaulis	L1	L1	L2
L. saturejoides ssp. *saturejoides*	L1	L2	L1
L. propinqua	L1	L2	L2

Supporting Information

Methods

Nomenclatural changes required

We included in the present paper two samples of the "*Linaria verticillata* group" [1]: *L. verticillata* ssp. *anticaria* (Boiss. & Reut.) L. Sáez & M.B Crespo – Spain: Málaga. El Torcal de Antequera, J.L. Blanco-Pastor (33JB09) – and *L. verticillata* ssp. *cuartanensis* (Degen & Hervier) L. Sáez & M.B. Crespo – Spain: Albacete. Yeste, P. F. Cannon et al. (2009.12.35 (RNG)). L. Sáez & M.B. Crespo [1]‡ considered that the "*Linaria verticillata* group" is formed by races that appeared as a result of geographical speciation from a southern Iberian common ancestor. As (i) taxonomic delimitation of this particular group has been controversial and (ii) non-monophyly was obtained for both samples used (see ITS and AGT trees in Fig. 1) such samples were treated here as independent taxa. Further, a possible hybrid origin or introgression was detected in the *L. verticillata* ssp. *cuartanensis* sample, thus not supporting such a simple geographical speciation model for this taxa as discussed by L. Sáez & M.B Crespo [1]. In consequence both samples of the "*Linaria verticillata* group" were herein circumscribed in a taxonomical rank at species level which we considered more appropriate: *L. anticaria* Boiss. & Reut. [2] was used instead *L. verticillata* ssp. *anticaria* whereas a nomenclatural change was required for *L. verticillata* ssp. *cuartanensis*:

L. cuartanensis (Degen & Hervier) Fern. Casas ex. Blanco-Pastor, **comb. et stat. nov.**

≡ *L. anticaria* ssp. *cuartanensis* Degen & Hervier in Bull. Acad. Int. Géogr. Bot. 15: 115 (1905) [3]‡ **[basionym]**

L. anticaria var. *cuartanensis* (Degen & Hervier) Degen in Bull. Acad. Int. Géogr. Bot. 16: 207 (1906)

L. cuartanensis (Degen & Hervier) Fern. Casas, in sched. 1976 (MA 348603, MA 348635), nom. inval.

Linaria verticillata ssp. *cuartanensis* (Degen & Hervier) L. Sáez & M.B. Crespo in Botanical Journal of the Linnean Society, 148: 239 (2005)

For the same reason as discussed above, a similar taxonomical delimitation (rank at species level) was applied for the "*Linaria alpina* group" formed by the samples *L. alpina* (L.) Mill. ssp. *alpina* – Spain: Huesca. Formigal, S. Martín Bravo (571SMB05) – and *L. alpina* ssp. *filicaulis* (Boiss. ex Leresche & Levier) M. Laínz – Spain: León. Pico Tres Provincias, C.M. Romero Rodríguez (769084 (MA)). Those samples were then considered as *L. alpina* (L.) Mill. and *L. filicaulis* Boiss. ex Leresche & Levier [4].

Further molecular analyses not performed here including additional samples would be valuable to establish a clear taxonomic circumscription of the "*Linaria verticillata"* and the *"Linaria alpina"* groups.

Coalescent Simulations

The analysis proceeded as follows: (1) Infer gene trees including branch lengths in MrBayes 3.1.2 using allele data. (2) Draw a pool of trees from the stable posterior distribution and convert these to chronograms in r8s 1.71. (3) Scale the chronograms into generations in Mesquite 2.6 using previous date estimates and generation time of the species. (4) Derive mutation rates per generation (μ) in cpDNA using the average number of pairwise differences (D_{xy}) between two species and their divergence time estimates. (5) Estimate allelic diversity (θ_w) in one species using DNAsp 5.10. (6) Derive the effective population size (N_e) via $\theta_w = 2\mu N_e$. (7) With the scaled chronograms and N_e, simulate under the coalescent new "gene trees" using the tool "coalescent contained within the current tree" in Mesquite 2.6. (8) Generate a distribution of tree to tree distances (symmetric distance in PAUP*) for each observed gene tree and corresponding simulated trees (baseline distribution), as well as among the gene trees (observed distribution), (9) Compare these distributions to determine whether significant differences exist among the observed gene trees in light of a lineage sorting null hypothesis.

Additional details for each step are now described.

For step 1, we used the haplotype trees (partial‡ AGT1 intron, ITS and cpDNA (rpl32-trnL^{UAG} and trnS-trnG intergenic spacers) inferred using MrBayes 3.1.2 [5].

For step 2, we drew 20 trees from the stable posterior distribution in order to account for uncertainty in our gene tree estimation. These trees were topologically representative of the complete Bayesian Analyses for each locus, being representative of the entire post-burn-in distribution of parameter values when compared using Tracer (i.e., produced overlapping 95% HPD estimates for log likelihood, posterior and other parameters)‡. We converted the trees into chronograms using the penalized likelihood function [6] implemented in r8s [7] in order to have branch lengths estimate time in each gene tree. Cross-validation to find the optimal smoothing parameter (10^k) was done using increments of k of 0.1, from k= -3 to 3 (using two random trees from the stable posterior distribution of each gene). Each chronogram was then trimmed to contain only a single allele from each individual, using Mesquite (Maddison and Maddison, 2006) to delete terminals while maintaining appropriate branch lengths. The alleles remaining were chosen to maximize topological incongruence among the loci, because several loci with alleles in the same clade do not introduce incongruence beyond lineage sorting and thus require no further explanation.

For step 3, chronograms obtained in r8s were based on a calibration point of the divergence between *Antirrhinum and Linaria* of 13.33 Ma (5% CI) (Vargas et al., unpublished). In Mesquite we scaled the branch lengths of the trees to convert them from units of time into units of generations. That was done by using the ultrametric trees, which implicitly assume equal generation times on all lineages, and setting the branches for annual species (and all branches in clades with only annuals) to have an "annual" scale (a generation time of one year). This assumes an early change to this state. The rest of the tree was treated as having the perennial state with a generation time of three years (generations divided by 3). Despite the impossibility in obtaining an ancestral state for annuality or perenniality (due to the incongruence among gene trees) with current methods, by doing this at least we could differentiate the generations scale (branch length scale) between the known annual species and the rest of the tree. By assuming perenniality in the rest of the tree we presented trees with shorter internal branches (fewer generations) for the coalescent simulations than if considering annuality, thus favoring the null hypothesis of lineage sorting alone to explain the gene tree incongruence (see below). In this way we are being conservative with respect to the null hypothesis‡.

The species having an "annual" scale were: *L. albifrons, L. flava, L. triphylla, L. laxiflora, L. warrionis, L. haelava, L. joppensis, L. arvensis, L. micrantha, L. simplex, L. propinqua, L. oblongifolia, L. saxatilis, L. bipunctata, L. tursica, L. glauca, L. saturejoides, L. bubanii, L. munbyana, L. filicaulis., L. badalii, L. amethystea, L. spartea, L. gharbensis, L. orbensis and L. chalepensis.*

For step 4, we calculated the mutation rate (μ_{gen}), where $\mu_{years}=D_{xy/sites/years}/2$ and μ_{gen} is obtained after scaling μ_{years}. We calculated the average number of pairwise differences (D_{xy}) between *L. elegans* (25 individuals from 25 different populations; trnS-trnG, rpl32-trnL and trnK-matK markers, Fernandez-Mazuecos, under review) and *L. cuartanensis* (1 individual, 1 population) using DNAsp [8]. Mutation rate per generation was obtained by dividing μ by the younger limit of the 95% HPD of divergence time (in years) between *L. elegans* and *L. cuartanensis* again, to favor‡ the null hypothesis.

For step 5: Theta per sequence [θw, an estimate of the population mutation rate under the neutral model; 9] was also estimated using DNAsp [8] over the 25 *L. elegans* individuals.

To explore the influence of N_e estimates on the coalescent simulations, we also calculated N_e using other two *Linaria* species. As the range size of a species seems to be highly correlated with its N_e [see examples in 10], we used two more species: *L. glacialis* (100 individuals from 10 populations, rpl32-trnL and rps16-trnK^{UUU}) and *L. simplex* (13 individuals from 13 populations, rpl32-trnL, trnS-trnG and trnL-trnF), with extreme lower and higher range sizes respectively. *Linaria elegans* occupies the northern part of the Iberian Peninsula, *L. glacialis* is a species that is restricted to a narrow altitudinal gradient on mountain and ridge tops in the Sierra Nevada range (Spain) and *L. simplex* is one of the few *Linaria* species with a widespread distribution, been present all over the Mediterranean basin.

For step 6, N_e was derived using $\theta_w = 2N_e\mu$ (2 for plastidial genes of diploid hermaphroditic species, [see 11]). We multiplied θ_w by 2 in order to apply the obtained values to nuclear sequence data. N_e values used in the coalescent simulations are shown in Supplementary Table 2‡.

For step 7, simulations on the set of chronograms were done in Mesquite assuming the above-mentioned N_e values for all species and their common ancestors, with 100 simulated trees produced for each input tree. Input trees were the 20 trees from the stable posterior distribution of each MrBayes analysis converted to chronograms in r8s and pruned to contain one allele (the most differentiated allele in respect to the other loci).

For step 8, nexus files were made that included the trees to be compared to one another and a text block (available upon request to the corresponding author)‡ describing an analysis of symmetric distance [12]. Each of the 20 trees drawn from the stable posterior distribution from the BA was placed at the beginning of the block of 100 simulated trees that arose by coalescent simulation. The first tree for each block was then compared to the remainder (using PAUP* commands "treedist metric=symdiff fromtree=1"). All 2000 distances generated this way (per locus) were pooled for subsequent steps. Distances obtained represented the "baseline distribution of tree distances". The 20 BA trees for each locus were pairwise compared to one another in a similar manner and also pooled for each pairwise comparison. Those distances represented the "observed distribution of tree distances". The distributions of distances were plotted in Excel (Microsoft). The null distribution is the distribution of values of the difference between the lower 95% HPD of the observed distribution and a critical value of the base line distributions (which depends on the number of loci used), under lineage sorting alone, and which was derived previously by simulation [13]. The critical value is chosen such that 5% of values of the null distribution fall above zero. However, the null distribution itself is not generated here.

For step 9, by doing pairwise comparisons, the null hypothesis of lineage sorting alone was rejected if none of the tree-to-tree distances (observed distributions), for a certain gene tree within their 95% HPD, overlapped the base line distribution of the gene used.

References‡

1. Sáez L, Crespo MB (2005) A taxonomic revision of the *Linaria verticillata* group (Antirrhineae, Scrophulariaceae). Botanical Journal of the Linnean Society 148: 229-244.
2. Boissier E, Reuter GF (1852) Pugillus plantarum novarum Africæ borealis Hispaniæque australis: Ex Typographia F. Ramboz et Socii.
3. Hervier J (1905) Excursions botaniques de M. Elisée Reverchon. Boulletin de l'académie internationale de géographie botanique 14e Anée (3e Série): 115.
4. Leresche LF, Levier E (1879) Decas Plantarum Novarum in Hispania Collectarium. Journal of botany, British and foreign 17: 200.
5. Ronquist F, Huelsenbeck JP (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572-1574.
6. Sanderson MJ (2002) Estimating Absolute Rates of Molecular Evolution and Divergence Times: A Penalized Likelihood Approach. Mol Biol Evol 19: 101-109.
7. Sanderson MJ (2003) r8s: Inferring absolute rates of molecular evolution and divergence times in the absence of a molecular clock. Bioinformatics 19: 301-302.
8. Rozas J, Sánchez-DelBarrio JC, Messeguer X, Rozas R (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics 19: 2496-2497.
9. Watterson GA (1975) On the number of segregating sites in genetical models without recombination. Theoretical Population Biology 7: 256-276.
10. Gossmann TI, Song B-H, Windsor AJ, Mitchell-Olds T, Dixon CJ, et al. (2010) Genome Wide Analyses Reveal Little Evidence for Adaptive Evolution in Many Plant Species. Molecular Biology and Evolution 27: 1822-1832.
11. Simon Joly, Bernard E. Pfeil, Bengt Oxelman, Patricia A. McLenachan, Peter J. Lockhart (2010) Correction. The American Naturalist 175: 621-622.
12. Penny D, Hendy MD (1985) The Use of Tree Comparison Metrics. Systematic Zoology 34: 75-82.
13. Maureira-Butler IJ, Pfeil BE, Muangprom A, Osborn TC, Doyle JJ (2008) The reticulate history of *Medicago* (Fabaceae). Systematic Biology 57: 466-482.

‡Changed from the published version

Manuscript 3

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Autecological traits determined two evolutionary strategies in Mediterranean plants during the Quaternary: low differentiation and range expansion versus geographical speciation in *Linaria*

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Abstract

The evolutionary patterns of the Mediterranean flora during the Quaternary have been relatively well documented based on phylogenetic and biogeographic analyses, but few studies have addressed the evolutionary traits that determined diversification and range expansion success during this period. We analysed previously published and newly generated sequences of three plastid noncoding regions (*rpl*32-*trn*L^{UAG}, *trn*S-*trn*G and *trn*L-*trn*F), the nuclear ribosomal internal transcribed spacer (ITS) and a low-copy nuclear gene intron (AGT1) of *Linaria* sect. *Supinae*, a group of angiosperms that diversified in the Quaternary. The origin and recent colonization dynamics of closely related lineages were inferred by biogeographic reconstruction and phylogeographic analyses, while breeding system experiments coupled with ecological and morphological data were used to test association with range expansion and diversification. A combination of traits, including selfing, short lifespan and the ability to tolerate a wide variety of substrates, were key factors underlying range expansion after long-distance dispersal throughout the Mediterranean basin. By contrast, self-incompatibility may have promoted higher diversification rates in narrow ranges of the Iberian Peninsula. We argue that a few traits contributed to the adoption of two contrasting strategies that may have been predominant in the evolution of Mediterranean angiosperms.

Keywords: breeding system, colonization, ecological requirements, Mediterranean, Quaternary, speciation

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Introduction

The complex palaeoclimatic and palaeoecological history of the Mediterranean basin has left a legacy of approximately 24 000 plant species (Thompson 2005). This outstanding biodiversity is primarily the result of diversification since the onset of the Mediterranean climate (3.4–2.8 Mya), which is suggested by palaeobotanical evidence (Palamarev 1989; Barrón *et al.* 2010; Jiménez-Moreno *et al.* 2010) and time-calibrated molecular phylogenies (Fiz-Palacios & Valcárcel 2013; Valente & Vargas 2013). After the onset of the Mediterranean climate, the subsequent Quaternary warm and cold episodes have been invoked to explain diversification splits of Mediterranean plant lineages revealed by molecular phylogenies, for example, *Anthemis* (clade II in Lo Presti & Oberprieler 2009), *Antirrhinum* (Vargas *et al.* 2009), *Aquilegia* (European clade in Bastida *et al.* 2010), *Cistus-Halimium* complex (Guzmán *et al.* 2009), *Erodium* (Fiz-Palacios *et al.* 2010), *Reseda* (sect. *Glaucoreseda*; Martín-Bravo *et al.* 2010) and *Linaria* (sect. *Supinae*; Blanco-Pastor *et al.* 2012). Recent climatic events have also been responsible for the diversification burst in exceptional plant radiations, such as *Dianthus* (Valente *et al.* 2010) and *Tragopogon* (Bell *et al.* 2012). Nevertheless, active diversification is only one of the patterns considered in relation to the Quaternary. Species persistence without differentiation (evolutionary stasis) or lineage extinctions are the alternative hypotheses

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invoked to explain the current species diversity in the Mediterranean (Kadereit *et al.* 2004; Willis & Niklas 2004; Postigo-Mijarra *et al.* 2010).

Key adaptations to colonization enhance the probability of persistence, thus evolutionary stasis coupled with range expansion might be considered to be an evolutionary strategy (Baker 1965; Wilson 1965). Despite extensive research on Quaternary species diffusion in the Mediterranean (e.g. Rodríguez-Sánchez *et al.* 2008; Guzmán & Vargas 2009; Escudero *et al.* 2010; Fernández-Mazuecos & Vargas 2010, 2011; Desamore *et al.* 2011; Lo Presti & Oberprieler 2011), the specific contributions of particular biotic and abiotic factors in determining range expansion remain unclear. Interactions between environmental conditions and intrinsic species' characteristics (autecological traits) have traditionally been put forward to explain successful range expansion in plant species (Brown 1996). Autecological traits include ecological requirements (Thompson *et al.* 1999; Fernández-Mazuecos & Vargas 2010), seed dispersal abilities (van der Veken *et al.* 2007; Gassó *et al.* 2009), lifespan (Pyšek *et al.* 2003; Cadotte *et al.* 2006; Caley *et al.* 2008) and breeding systems (Baker 1955, 1967; Stebbins 1957; Busch 2005; Randle *et al.* 2009). Species with greater seed dispersal ability, higher reproductive rate and ample ecological requirements are considered to be more likely to persist and expand into new regions under changing climatic conditions (Angert *et al.* 2011). Information on the particular characteristics of individual species is available from taxonomy (e.g. seed dispersal structures, lifespan, substrate requirements) and pollination experiments (e.g. breeding system). Nevertheless, associations between single key traits and range expansion have been inconsistent in the literature (Table 1). Moreover, autecological traits may not only affect range expansion, but also speciation and extinction processes (i.e. diversification; Table 1). Therefore, the net contribution of each key trait in the mode of evolution of plant lineages remains unclear.

A wide variety of autecological traits have been reported in *Linaria* sect. *Supinae* (hereafter *Supinae*), a group of angiosperms that diversified during the Quaternary (Blanco-Pastor *et al.* 2012). *Supinae* species have highly variable range sizes: most of the species (40) are narrow endemics, three species (*L. simplex*, *L. arvensis* and *L. micrantha*) are distributed throughout the Mediterranean basin and one (*L. alpina*) occurs in Alpine regions across Europe (Sutton 1988) (see Fig. 1). The differentiation of *Supinae* was closely related to the establishment of the Mediterranean climate and to the Pleistocene climatic oscillations (Blanco-Pastor *et al.* 2012). The 44 species therefore form an appropriate

Table 1 Association of four plant characteristics with range expansion and/or diversification success as suggested in studies published in the last decade

	Range expansion success		Diversification success	
Plant characteristics	Association	References	Association	References
Selfing	+	Rambuda & Johnson (2004)	−	Goldberg *et al.* (2010)
	+	Schueller (2004)	−	Ferrer & Good (2012)
	−	Sutherland (2004)		
	+	Busch (2005)		
	−	Lowry & Lester (2006)		
	ø	Stout (2007)		
	+	van Kleunen & Johnson (2007)		
	+	Randle *et al.* (2009)		
	+	Rodger *et al.* (2013)		
Short generation time	+	Pyšek *et al.* (2003)	+	Petit & Hampe (2006)
	−	Sutherland (2004)	−	Drummond (2008)
	+	Cadotte *et al.* (2006)	+	Smith & Donoghue (2008)
	+	Caley *et al.* (2008)	+	Smith & Beaulieu (2009)
	ø	Gassó *et al.* (2009)		
Seed dispersal structures	+	Cadotte *et al.* (2006)		
	+	van der Veken *et al.* (2007)		
	+	Gassó *et al.* (2009)		
	ø	Lake & Leishman (2004)		
Wide variety of substrates	+	Lyford *et al.* (2003)	−	Verboom *et al.* (2004)
	+	Cadotte *et al.* (2006)	−	Alvarez *et al.* (2009)
	+	Dullinger *et al.* (2012)		

Association codes: + positive association, − negative association, ø no association.

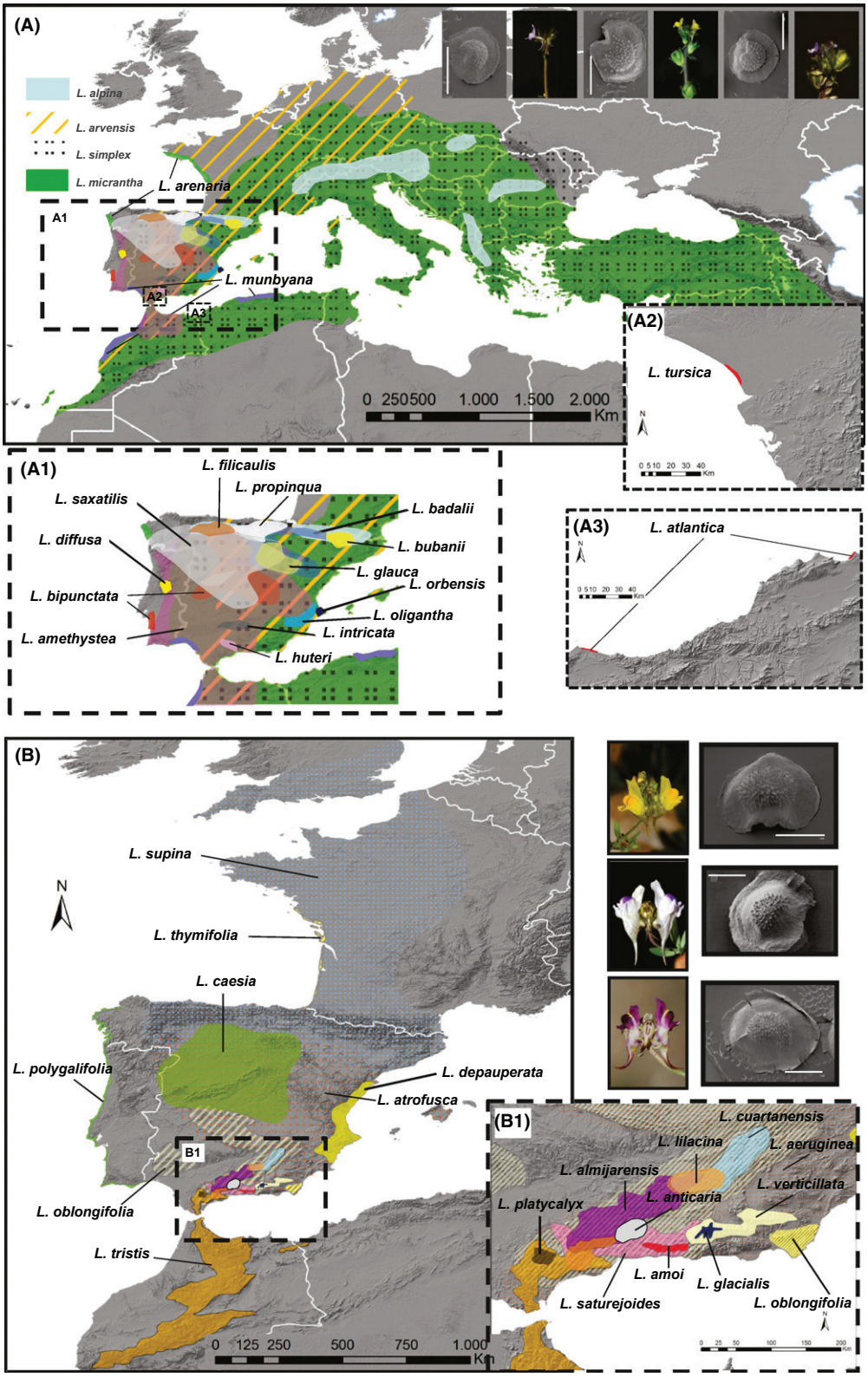

Fig. 1 Geographical distribution of *Linaria* sect. *Supinae* species. Distribution range of (A) *ssSax+ssArv* clade *and* (B) *ssSup clade* (clade names in Fig. 2). Insets show representative seed photographs using scanning electron microscopy and flower images of (A) *ssArv* and (B) *ssSup* species. White lines on seed photographs indicate the scale (1 mm). Information about the geographical distribution was obtained from GBIF webpage (http://www.gbif.org), ANTHOS webpage (http://www.anthos.es) and Valdés (1970a).

group in which to test the effects of specific traits on both diversification and range expansion dynamics during the Quaternary.

Three particular objectives were addressed in this study. We aimed: (i) to date evolutionary events by extending a previous phylogeny of *Supinae* (Blanco-Pastor *et al.* 2012), including additional species, populations and DNA regions (ii) to infer colonization patterns based on ancestral area reconstruction and haplotype networking analyses; and (iii) to determine the association of species' traits with range expansion and diversification success. The ultimate goal was to contrast key traits responsible for divergent evolutionary patterns in the Mediterranean during the Quaternary.

Materials and methods

Study group, sampling strategy and DNA sequencing

The section *Supinae* of genus *Linaria* includes 44 species that have a primarily circum-Mediterranean distribution, and the highest diversity (40 species) is found in the Iberian Peninsula (Sutton 1988). Most of the species of this section are characterized by their laterally compressed seeds with an encircling wing (see Fig. 1), a presumed adaptation to wind dispersal (Elisens & Tomb 1983; but see Nadeau & King 1991). Twenty-seven of the 44 species are annuals, 10 perennials and seven can be annuals or perennials. Breeding systems of *Supinae* species vary from strictly self-fertilizing species to species that are obligate out-crossers (Valdés 1970b; Valdés & Cabezudo 1977).

We sampled a total of 120 individuals of *Supinae* species and the out-group (see Table S1, Supporting information). We applied species delimitation as in Blanco-Pastor *et al.* (2012), which basically follows Sutton's delimitation (1988) for the non-Iberian species and Sáez & Bernal's delimitation (2009) for the Iberian species. We additionally considered the species *L. atrofusca* (Rouy 1883) as an exploratory analysis carried on plastid sequences supported separation for this taxon from *L. aeruginea* ssp. *aeruginea* samples. In total, this study included 37 of the 44 *Supinae* species considered by Sutton (1988). The out-group was formed by one species of *Chaenorhinum*, one of *Antirrhinum* and 13 *Linaria* species representing the remaining seven sections of *Linaria* following Fernández-Mazuecos *et al.* (2013): sect. *Macrocentrum* (*L. chalepensis*), sect. *Pelisserianae* (*L. triornithophora*), sect. *Versicolores* (*L. elegans*, *L. gharbensis*, *L. multicaulis*, *L. incarnata*, *L. spartea*), sect. *Linaria* (*L. vulgaris*), sect. *Speciosae* (*L. nivea*, *L. repens*, *L. purpurea*), sect. *Diffusae* (*L. reflexa*) and sect. *Lectoplectron* (*L. texana*). One individual per population was sampled, while the number of populations per species varied upon species distributions and the DNA regions sequenced (see Table S1, Supporting information).

All individuals were collected in the field and dried in silica gel or obtained from herbaria (MA, SEV, RNG). Total genomic DNA was extracted using Dneasy Plant Mini Kit (QUIAGEN Inc., CA, USA). Standard primers were used for amplification of three plastid intergenic spacers *rpl*32-*trn*L^{UAG} (Shaw *et al.* 2007), *trn*S^{GCU}-*trn*G^{UUC} (Hamilton 1999) and *trn*L^{UAA}-*trn*F^{GAA} (Taberlet *et al.* 1991), the nuclear ribosomal internal transcribed spacer (ITS; White *et al.* 1990) and the low-copy nuclear gene intron AGT1 (Liepman & Olsen 2001). One hundred and seventy-four plastid and nuclear (*rpl*32-*trn*L, *trn*S-*trn*G, ITS, AGT1) sequences (counting unphased data) were obtained from Blanco-Pastor *et al.* (2012), Fernández-Mazuecos *et al.* (2013) and Blanco-Pastor *et al.* (2013). The remaining 312 sequences were newly generated following the same amplification and sequencing procedures as in Blanco-Pastor *et al.* (2012). Newly generated sequences were submitted to the GenBank (see Tables S1 and S2, Supporting information).

Species tree inference

Phylogenetic incongruence was previously found in the study group (*Supinae*), due to both incomplete lineage sorting and hybridization/introgression (Blanco-Pastor *et al.* 2012). Fine-scale detection and incorporation of reticulation processes to character mapping would lead to more realistic reconstructions in the future. Nevertheless, no standard methods are currently available for the accurate reconstruction of reticulate phylogenies using scarce number of loci (see discussion in Blanco-Pastor *et al.* 2012). A number of coalescent-based methods have been recently proposed for the inference of species trees that account for incongruence between gene trees caused by incomplete lineage sorting (for review see Liu *et al.* 2009). Here, we employed a set of ptDNA (the concatenated *rpl*32-*trn*L^{UAG}, *trn*S-*trn*G and *trn*L-*trn*F regions), ITS and AGT1 sequences, including more than one individual per taxon in most cases (see Table S1, Supporting information) and analysed the data under the multispecies coalescent method of *BEAST (Heled & Drummond, 2010), implemented in BEAST 1.7.3. To avoid strong violations of assumptions of the *BEAST model and to help convergence of chains, we first excluded from the analyses the putative hybrid/introgressed species originated by the cross of the most distant *Supinae* lineages (*L. orbensis*, *L. oblongifolia*, *L. saturejoides*) as observed in Blanco-Pastor *et al.* (2012). One more species (*L. huteri*) was also excluded because the finding of a similar incongruent signal in gene trees. With this approach, we depicted the

evolution of *Supinae* in a tree-like fashion, fitting the incongruent signals under the assumptions of the multispecies coalescent model. An enforcement of a tree-like phylogeny was made; however, the phylogenetic uncertainty reflected by the signal of hybridization or low phylogenetic resolution obtained with *BEAST was further incorporated in the subsequent analyses that used the phylogeny as input (trees from the stable posterior distribution, see below). Further analysis details are indicated in Appendix S1 (Supporting information).

Biogeographic reconstruction

We conducted biogeographic analyses in order to reconstruct the origin and directionality of range expansions within *Supinae*. As the temporal origin of this group was estimated in the late Pliocene–early Pleistocene (Blanco-Pastor *et al.* 2012), and the Mediterranean geography has not suffered major changes since then, five current areas were considered for the biogeographic analyses based on the distribution of *Supinae* taxa and significant barriers, such as marine water bodies and high mountains. The five areas were (i) North-West Africa, (ii) Iberian Peninsula, (iii) central Europe, (iv) Italian and Balkan Peninsulas and (v) Anatolia. We allowed a maximum of five areas in ancestral ranges according to the current maximum species ranges.

We analysed the data set with statistical-DIVA (S-DIVA), a Bayesian approach to the parsimony based method dispersal–vicariance analysis (DIVA; Ronquist 1997). The use of a Bayesian method, coupled with a dispersal–vicariance analysis, provides a reliable procedure in biogeographic analyses when coping with phylogenetic uncertainty (Nylander *et al.* 2008; Harris & Xiang 2009), as occurred in the present study. S-DIVA analyses were conducted following the method of Harris & Xiang (2009) implemented in the program RASP 2.1b (Yu *et al.* 2010, 2011), which accounts not only for phylogenetic uncertainty but also for area uncertainty in DIVA optimization. S-DIVA analysis was conducted using 10 000 random trees after the burn-in period from the *BEAST run and the MCC tree as the final tree. All trees were previously pruned to contain only the monophyletic sect. *Supinae*.

Haplotype networks

Plastid markers are known to be one of the most reliable markers for inferring plant colonization by seed dispersal (Schaal *et al.* 1998; see Fernández-Mazuecos & Vargas 2011 for an example in *Linaria*). The ptDNA genome is structurally stable, haploid, nonrecombinant and maternally inherited in *Linaria* (Corriveau & Coleman 1988). To infer colonization patterns at the regional scale, we additionally performed phylogeographic analyses of the ptDNA haplotypes of closely related species through a network approach. Haplotype network analyses were conducted for the three main ptDNA *Supinae* lineages separately (*ssArv*, *ssSax* and *ssSup*; see Appendix S1, Fig. S1, Supporting information) because when the full data set was analysed, the haplotype network was disconnected. Genealogical relationships among haplotypes were inferred using statistical parsimony implemented in TCS v1.21 (Clement *et al.* 2000). The maximum number of differences resulting from single substitutions among haplotypes was calculated with 95% confidence limits treating gaps as missing data.

Breeding systems

To estimate breeding systems of *Supinae* species, we first obtained data of 15 species from previous studies (Table S4, Supporting information). Additionally, we cultivated and studied in the field plants of 18 species and performed typically three treatments: self-pollination (spontaneous autogamy; SA), hand self-pollination (forced autogamy; FA) and hand cross-pollination (forced xenogamy; FX). Additionally, we calculated pollen/ovule (P/O) ratios in 15 species (see Appendix S1, Supporting information). In SA, flowers were nonmanipulated; in FA, we forced the spread of mature pollen grains of the stamens onto the stigma of the same flower; in FX, before the anthesis, flowers were emasculated, and after anthesis, we spread mature pollen grains from a different individual onto the stigma of the flower. We tested for each species four to 40 individuals and four to 101 flowers per treatment. We failed in cultivating eleven species, namely *L. atlantica*, *L. badalii*, *L. bipunctata*, *L. bubanii*, *L. cuartanensis*, *L. diffusa*, *L. glauca*, *L. intrincata*, *L. munbyana*, *L. propinqua* and *L. thymifolia*. In summary, previous studies and our experiments allowed categorizing the breeding system of 28 of the 39 species analysed.

Species traits associated with range expansion

In order to test potential association of species traits and range expansion, we applied a Bayesian approach to the stochastic mapping of character states in a phylogeny and estimated correlation statistics using posterior predictive *P* values (Huelsenbeck *et al.* 2003) as implemented in SIMMAP v.1.5 (Bollback 2006). This approach accounts for uncertainty in the process of character change, the phylogenetic history and the character states at tips. We analysed four traits that have been historically hypothesized as important for range expansion: (i) breeding system, (ii) lifespan, (iii) seed

type and (iv) substrate requirements. Character states were coded as binary data following the information shown in Table S6 of Supporting information. A species was considered to have high range expansion ability when distributed in >2 areas as delimited in the biogeographic analysis. See Appendix S1 (Supporting information) for further analysis details.

In order to visualize the evolution of character states associated with range expansion, we additionally performed ancestral state reconstruction (hereafter ASR) for such characters: (i) breeding system, (ii) lifespan and (iii) substrate requirements (see below). We reconstructed characters using a maximum-likelihood method implemented in Mesquite (Maddison & Maddison 2009). We selected the single-parameter Markov k-state model (MK1) (Lewis 2001), thus assigning equal probability to any state of the character. The best estimate of character state for each node in each of the trees was determined using the likelihood ratio test with a decision threshold of two, which was chosen in order to make a conservative assignation of character states at nodes (Maddison & Maddison 2009). Reconstructions were considered equivocal if the difference in log-likelihood between alternative reconstructions was below this threshold. To account for tree topology uncertainty, character optimizations were repeated for each of 10 000 trees from the posterior distribution of *BEAST analysis and summarized in the MCC tree.

Species traits associated with diversification

We estimated the effect of species traits on *Linaria* diversification (speciation *minus* extinction) using the binary-state speciation and extinction (BiSSE) model (Maddison *et al.* 2007) implemented in the package *diversitree* v.0.7-2 (Fitzjohn *et al.* 2009) of R software (Available at: http://www.R-project.org). The four characters analysed for associated diversification were the same as indicated in the previous section. We used the MCC tree, but nodes with posterior probability below 0.2 were collapsed to account for hard polytomies. In order to explore the effect of phylogenetic uncertainty, we also analysed 10 noncollapsed post-burn-in trees from the stable posterior distribution of the *BEAST analysis. We compared state-dependent diversification models against nested models with parameters (speciation, extinction or transition rate) constrained to be equal for both states. For that, we contrasted maximum-likelihood parameter values of the unconstrained model (full BiSSE model, 6 parameters) vs. constrained models (5 parameters). We tested the significance of model differences by performing likelihood ratio tests comparing $-2\Delta\ln L$ to a Chi-squared distribution. See Appendix S1 (Supporting information) for further analysis details.

Results

Species tree inference and lineage divergence times

The *BEAST species tree analysis presented in this study (Fig. 2) extends the previously known phylogenetic information of *Supinae* (Blanco-Pastor *et al.* 2012). The analysis revealed two main clades: one clade contained subsect. *Supinae* (*ssSup*) species and the other contained subsect. *Saxatile* and subsect. *Arvenses* (*ssSax+ssArv*) species as obtained in the multilabelled species tree of Blanco-Pastor *et al.* (2012). The *BEAST species tree herein presented placed the diversification of *Supinae* lineages in the Quaternary with slightly more recent divergence times than the ones obtained in Blanco-Pastor *et al.* (2012), which may be a consequence of the inclusion of plausible hybrid/introgressed species in the *BEAST analysis (Leaché *et al.* 2013). Lineage-through-time (LTT) plots (Fig. 2) clearly depicted diversification *Linaria* sect. *Supinae* occurring primarily during the last 0.5 Ma.

Biogeographic reconstruction and haplotype networks

The S-DIVA analysis recovered the *Supinae* most recent common ancestor (MRCA) in the Iberian Peninsula (IP) (B: 70.21%; Fig. 3A). This ancestor diversified in two sister lineages also present in the IP: *ssSup* MRCA (B: 78.16%) and *ssSax+ssArv* MRCA (B: 68.68%). The MRCAs of *ssArv* and *L. micrantha* + *L. simplex* were estimated to be exclusively present in the IP (Fig. 3A). The MRCA of *L. filicaulis* and *L. alpina* was also reconstructed in the IP (B: 100%).

Three independent phylogeographic analyses were conducted for the three ptDNA lineages: (i) *ssArv*, (ii) *ssSax* and (iii) *ssSup* (Fig. 4, see also Fig. S1 and Table S3, Supporting Information). The *ssArv* ptDNA lineage showed a poor geographical structure and low differentiation among distant populations: 10 of the 14 sampled haplotypes were exclusive to a single population, but one haplotype (haplotype 10) was shared by Iberian populations and three haplotypes (haplotypes no. 1, 4, 11) were shared by separate populations (Fig. 4A and Table S3, Supporting Information). The *ssSax* and *ssSup* ptDNA networks showed moderate-to-strong geographical structure and high differentiation among close populations (Fig. 4B–C and Table S3, Supporting Information). The *ssSax* network was formed by 23 sampled haplotypes, of which 19 were exclusive to a single population and four were shared by nearby populations and species. Five haplogroups were found in *ssSax*, three of them showing a moderate geographical structure (Fig. 4B and Table S3, Supporting Information). The *ssSup* ptDNA network showed 31 present haplotypes,

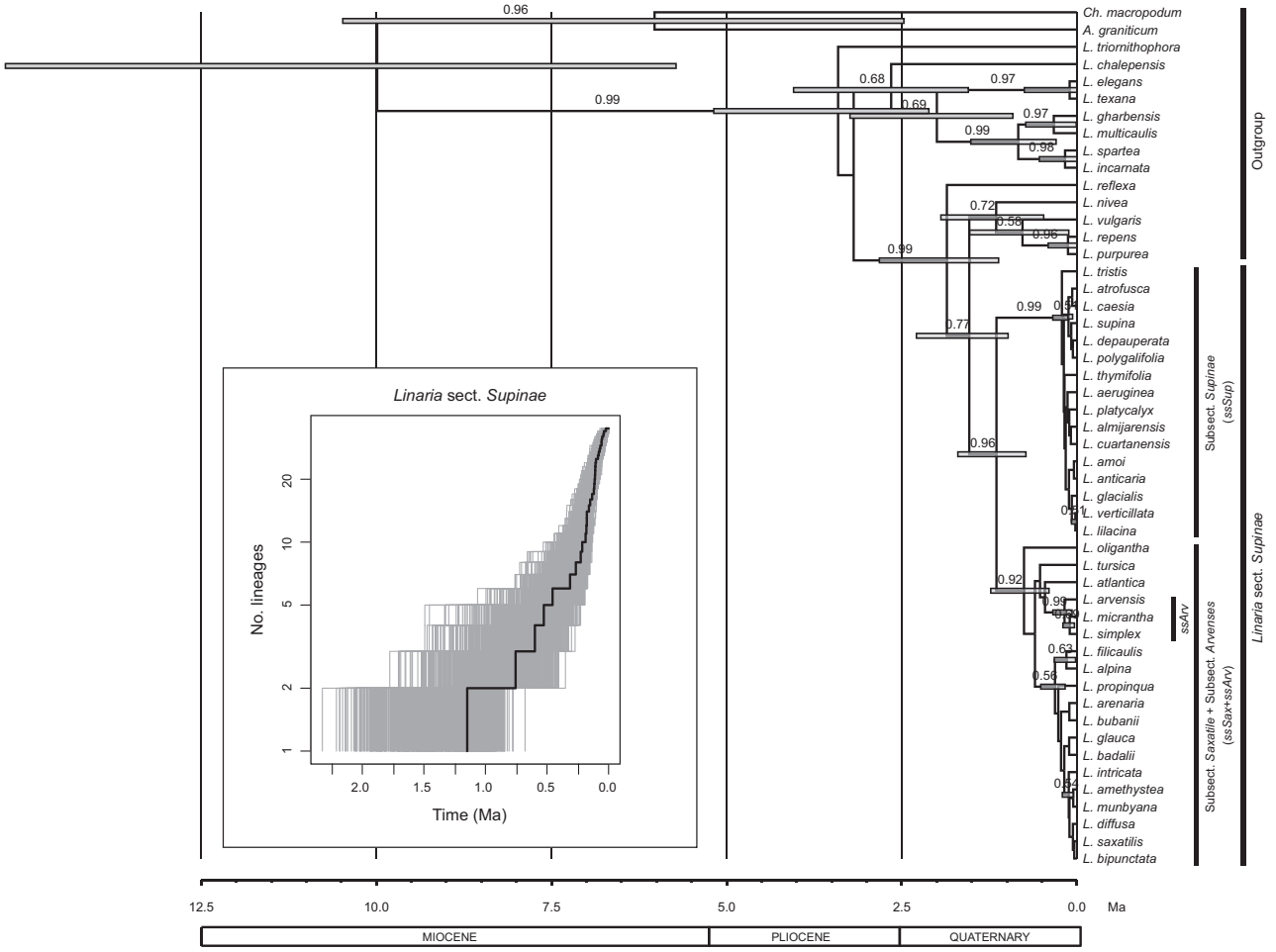

Fig. 2 Dated phylogeny of *Linaria* sect. *Supinae*. The *BEAST maximum clade credibility tree is shown with 95% of highest posterior density intervals for divergence times of clades with posterior probability above 0.5. Numbers above branches indicate Bayesian posterior probabilities. Inset show a lineage-through-time plot of *Linaria* sect. *Supinae*.

of which 25 were exclusive to a single population and six were shared by geographically close populations and species. Four out of five haplogroups showed a strong geographical structure (Fig. 4C and Table S3, Supporting Information).

Breeding systems

We obtained breeding system data from literature and own experiments. Estimates of the Cruden's test using P/O ratios were congruent with the results of the three manipulation treatments for 13 of the 14 species tested (see Appendix S1 and Table S4, Supporting information). Only in one species (*L. glacialis*) P/O ratios and manipulation treatments showed inconsistent results. The three *ssArv* species were recognized as selfers, because they produced high values of fruit/flower ratios in the spontaneous autogamy treatment (SA: 0.571–0.960; see Table S4). In contrast, the *ssSax* and *ssSup* species were categorized as outcrossers, except for *L. glacialis*, *L. tursica*, *L. arenaria* and one population of *L. alpina*; see Table S4 (Supporting information). The *ssSax* and *ssSup* species analysed here yielded very low values in the autogamy treatments (except for *L. glacialis*), but high values of fruit/flower ratios in the forced xenogamy treatment (SA: 0–0.164; FA: 0–0.235; FX: 0.333–1; see Table S4).

Traits associated with range expansion and diversification

Phylogenetic correlations between range expansion and species traits, based on SIMMAP character state correlation *d* and *m* statistics, are summarized in Table 2 (see also Table S7). Range expansion ability was associated with breeding systems, lifespan and substrate requirements and positively correlated with autogamy, annuality and high tolerance to diverse substrates in *m* and *d* statistics, whereas seed type turned out to be nonsignificant (Table 2).

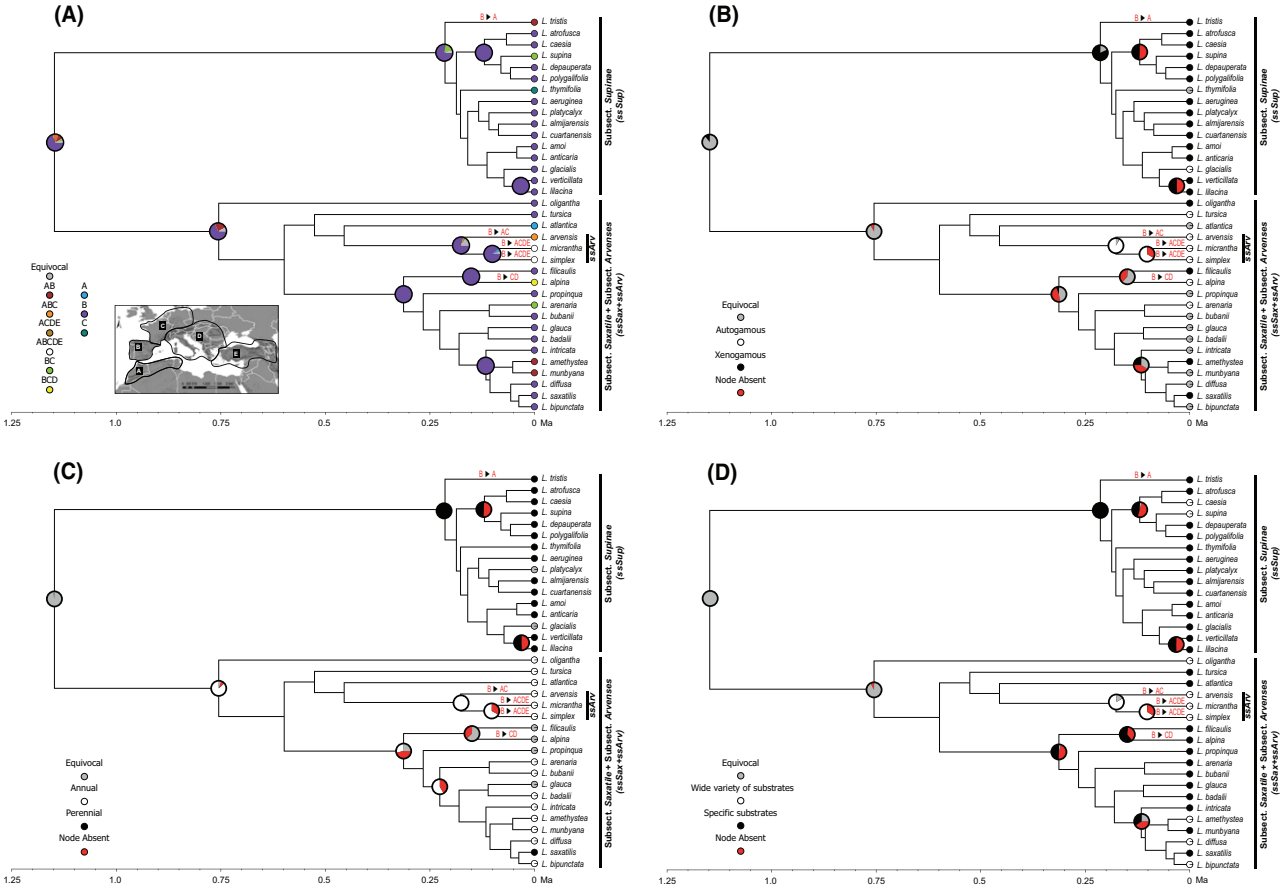

Fig. 3 (A) Biogeographic reconstruction of *Linaria* sect. *Supinae*. Ancestral areas derived from the statistical dispersal–vicariance analysis (S-DIVA). Optimizations were done over 10 000 pruned trees randomly sampled from the *BEAST run, as implemented in the program RASP 2.1b. Pie charts represent marginal probabilities of ancestral areas (the summary of area reconstructions over all sampled trees for each node using the pruned *BEAST MCC tree as reference). Grey sections of pie charts represent hidden probabilities (ranges with probabilities less than 5%). (B–D) Maximum-likelihood character optimization analyses performed in Mesquite. It is shown the ancestral state reconstruction of characters associated with range expansion ability as determined by SIMMAP analyses: (B) breeding system, (C) lifespan and (D) substrate requirements. Character optimizations were repeated for each of 10 000 pruned trees randomly chosen from the stable distribution of *BEAST analysis. Each chart shows the percentage of trees for which a given state was reconstructed as ancestral for that node. Grey colour at nodes represents the percentage of equivocal reconstructions; red colour at nodes represents percentage of trees with the node absent.

Ancestral state reconstructions under maximum-likelihood (integrating over 10 000 topologies) for breeding system, lifespan and substrate requirements showed a xenogamous ancestor for *ssSup* and an equivocal reconstruction for *ssSax+ssArv*, whereas an autogamous ancestor was obtained for *ssArv* (Fig. 3B). A perennial ancestor was recovered for *ssSup*, and an annual ancestor was recovered for *ssSax+ssArv* and *ssArv* (Fig. 3C). The analysis also showed a *ssSup* ancestor with low tolerance to different substrates, a *ssSax+ssArv* ancestor with equivocal reconstruction and a *ssArv* ancestor with high tolerance to different substrates (Fig. 3D).

An effect of breeding system on speciation rates was supported by the likelihood ratio test of BiSSE models. We detected significant differences between the full model and a BiSSE model with constrained speciation for the MCC tree and seven out of 10 randomly chosen trees. No effect on extinction rates or character transition rates was detected (Table 3). The MCMC-BiSSE analysis that accounted for uncertainty in parameter estimation and phylogenetic relationships revealed higher mean values of speciation and net diversification rates for xenogamy than for autogamy but with overlapping 95% credibility intervals (Fig. 5 and Table 4) (see also Fig. S2, Supporting information).

Discussion

Iberian origin of Supinae lineages in the Quaternary

Whether a species becomes established is determined, in part, by where and when the species originated

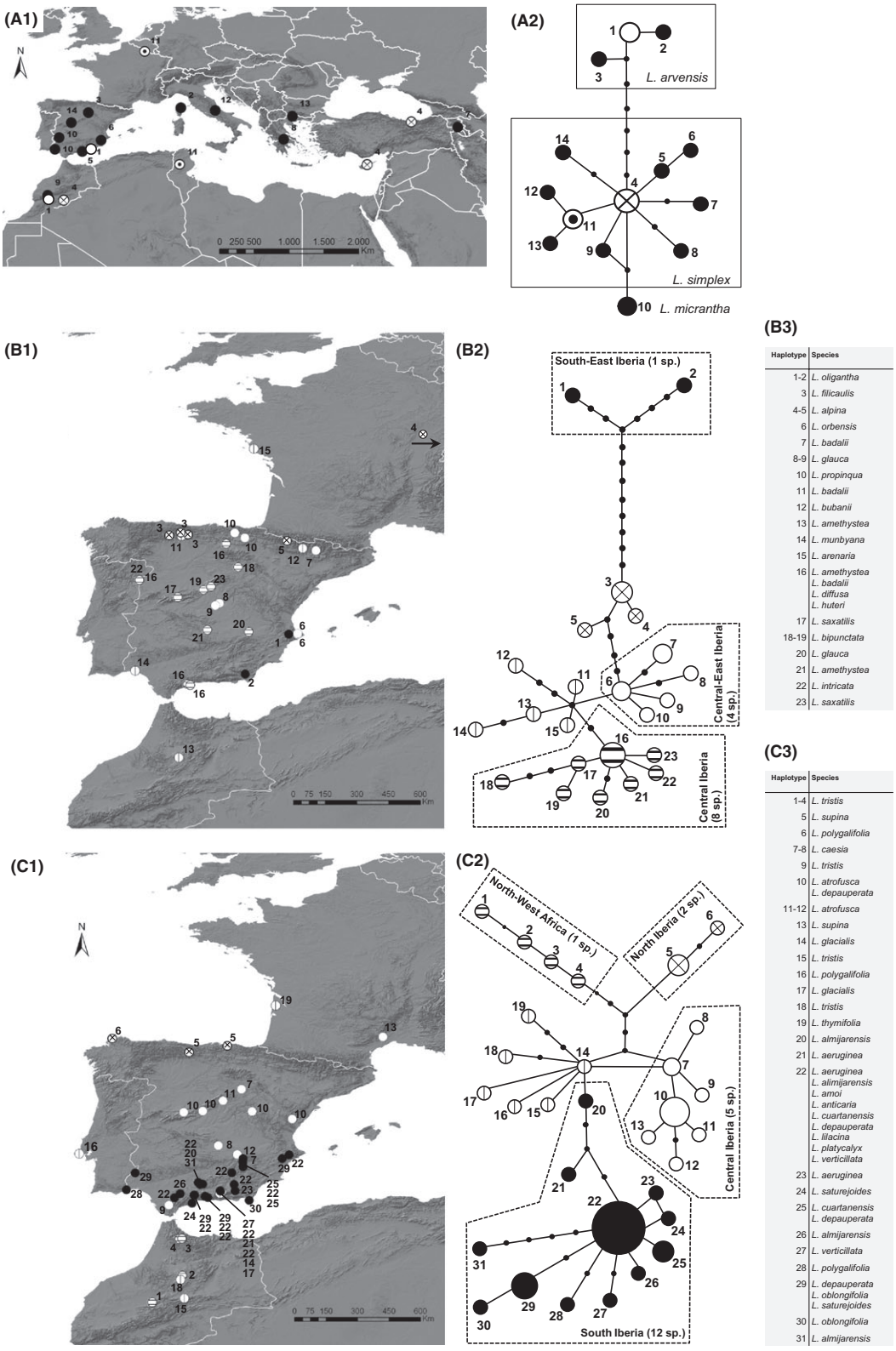

Fig. 4 *Linaria* sect. *Supinae* plastid lineages (A) *ssArv*, (B) *ssSax* and (C) *ssSup*. It is shown the plastid haplotypes distribution (A1, B1 and C1), the statistical parsimony networks (A2, B2 and C2) and the species names linked to plastid haplotypes (A2, B3 and C3). Lines represent single mutational steps; black small circles are haplotypes extinct or not found (missing haplotypes). See also the phylogenetic relationships of plastid lineages in Fig. S1 of Supporting information.

Table 2 Character correlation *m* and *d* statistics obtained with SIMMAP (Bollback 2006). Correlation statistics among range expansion and binary species traits within *Linaria* sect. *Supinae* are shown

	Breeding system		Lifespan		Seed type		Substrate requirements	
	Autogamous	Xenogamous	Annual	Perennial	Small wingless seeds	Large winged seeds	Wide variety of substrates	Specific substrates
High range expansion ability								
m Statistic	0.06 (0.04*)	−0.01 (NS)	0.03 (0.03*)	5×10^{-5} (NS)	2×10^{-4} (NS)	7×10^{-4} (NS)	7×10^{-4} (0.04*)	-4×10^{-4} (0.05*)
d Statistic	0.03 (0.05*)	−0.03 (0.05*)	0.03 (0.04*)	−0.03 (0.04*)	-1×10^{-4} (NS)	1×10^{-4} (NS)	5×10^{-4} (0.04*)	-5×10^{-4} (0.05*)

Correlation statistics: *d*, differences between the observed and expected frequency of co-occurrence of states on the phylogeny; *m*, fraction of the phylogeny one state is associated with another (Huelsenbeck *et al.* 2003; see also http://www.simmap.com/pgs/stats_corr.htm).
Significance codes: NS, not significant; *≤0.05.

(Stebbins 1950; Lonsdale 1994; Reichard & Hamilton 1997; Kolar & Lodge 2001). The dating analysis of Blanco-Pastor *et al.* (2012) and the analysis presented here (Fig. 2) indicate that *Supinae* lineages diversified during the Quaternary, after the establishment of the Mediterranean climate regime (2.8 Ma, Suc 1984). The ancestral range reconstruction analysis placed the origin of all disjunction events in the Iberian Peninsula. Ample differences in range sizes and clade richness were found in a narrow spatiotemporal framework. This provided a valuable opportunity to investigate characters promoting contrasting range expansion and diversification under similar conditions.

Differential colonization in Supinae

Despite the common spatiotemporal origin of *Supinae* species (Quaternary, Iberian Peninsula; Fig. 3A), four species (*L. arvensis*, *L. micrantha*, *L. simplex* and *L. alpina*) colonized territories over long distances throughout the Mediterranean basin and Europe, whereas 31 species remained restricted to the western Mediterranean region (see Fig. 1). Biogeographic reconstruction (Fig. 3A) supported the occurrence of at least four successful long-distance dispersal (LDD; >10 km) events across the Mediterranean Sea during the colonization of northern Africa by *L. tristis*, *L. arvensis*, *L. micrantha* and *L. simplex*. One or two more LDD events across the western Mediterranean may have occurred as evidenced by the presence of *L. amethystea* and *L. munbyana* in southern Iberia and northern Africa.

Although every species has distribution peculiarities, two distinct patterns of diffusion were found during the Quaternary. Phylogeographic reconstruction showed that *ssSax* and *ssSup* ptDNA lineages differentiated geographically in narrow ranges of the Iberian Peninsula (Fig. 4B–C). Limitations of *ssSax* and *ssSup* species to LDD or establishment may explain this pattern and the absence of all species in central and eastern Mediterranean. Conversely, a more dynamic colonization was inferred for the *ssArv* lineage (Fig. 4A). The lack of geographical structure and the presence of two derived plastid haplotypes (1 and 11) in two areas separated by the Mediterranean Sea (Iberia-northwestern Africa and central Europe-northwestern Africa) suggest the occurrence of LDD events in two distinct areas of the Mediterranean basin (Fig. 4A): the Strait of Gibraltar and the central Mediterranean. Such connections have been previously detected for other angiosperms and are associated with the short distances between Europe and Africa in these areas that were even shorter during glacial periods (Rodríguez-Sánchez *et al.* 2008; Guzmán & Vargas 2009; Fernández-Mazuecos & Vargas 2010, 2011).

Table 3 Estimation of character states associated with shifts in speciation, extinction and character transition rates. Model parameters were estimated by maximum-likelihood values of the unconstrained model (full BiSSE model, 6 parameters) and models with parameters to be equal for both states (5 parameters). The collapsed MCC tree was used to indicate the parameter values, lnLik and AIC of the models. Significance of model differences were calculated by performing likelihood ratio tests comparing $-2\Delta\ln L$ to a Chi-squared distribution. Significance of model differences was also calculated for 10 random trees from the stable posterior distribution of the *BEAST analysis

Character	Model	d.f.	λ_0	λ_1	μ_0	μ_1	q_{01}	q_{10}	lnLik	AIC	χ^2	Pr(>|Chi|) (No. trees with $P < 0.05$)
A—Breeding system	Unconstrained	6	1.77	5.24	4.74×10^{-9}	5.07×10^{-9}	1.01	8.93×10^{-1}	−0.66	13.33	—	—
	Symmetric speciation (λ_0–λ_1)	5	3.94		2.59	1.08×10^{-9}	8.44×10^{-1}	1.25	−2.46	14.92	3.59	0.05* (7/10)
	Symmetric extinction (μ_0–μ_1)	5	1.77	5.24	1.05×10^{-8}		1.01	8.93×10^{-1}	−0.66	11.33	1.11×10^{-8}	0.99(NS) (0/10)
	Symmetric transition rate (q_1–q_{10})	5	1.74	5.22	4.65×10^{-6}	7.31×10^{-6}	9.91×10^{-1}		−0.67	11.35	0.01	0.89(NS) (0/10)
B—Lifespan	Unconstrained	6	3.60	4.70	1.59	8.32×10^{-6}	2.67×10^{-1}	6.20×10^{-7}	1.95	8.08	—	—
	Symmetric speciation (λ_0—λ_1)	5	4.32		2.60	1.21×10^{-7}	2.29×10^{-1}	3.09×10^{-6}	1.81	6.36	0.27	0.59(NS) (1/10)
	Symmetric extinction (μ_0—μ_1)	5	2.87	4.69	2.02×10^{-4}		3.58×10^{-1}	1.56×10^{-6}	1.74	6.50	0.41	0.52(NS) (0/10)
	Symmetric transition rate (q_1—q_{10})	5	3.70	4.91	1.72	1.04×10^{-8}	2.04×10^{-1}		1.11	7.76	1.67	0.19(NS) (0/10)
C—Seed type	Unconstrained	6	3.33×10^{-8}	4.24	2.24×10^{-6}	4.85×10^{-1}	2.07×10^{-1}	3.47×10^{-1}	5.88	0.22	—	—
	Symmetric speciation (λ_0—λ_1)	5	4.42		4.32	4.99×10^{-7}	4.22×10^{-1}	3.93×10^{-6}	6.15	−2.31	0.00	1.00(NS) (5/10)
	Symmetric extinction (μ_0—μ_1)	5	1.14	4.87	4.00×10^{-9}		9.25×10^{-1}	3.09×10^{-7}	7.89	−5.79	0.00	1.00(NS) (1/10)
	Symmetric transition rate (q_1—q_{10})	5	1.21×10^{-6}	4.22	1.13×10^{-6}	4.02×10^{-1}	3.69×10^{-1}		5.87	−1.75	0.01	0.90(NS) (0/10)
D—Substrate requirements	Unconstrained	6	1.22	4.40	3.30×10^{-9}	5.06×10^{-8}	2.54	1.74	−11.13	34.259	—	—
	Symmetric speciation (λ_0—λ_1)	5	4.20		6.76	2.43×10^{-8}	3.61×10^{-1}	2.10	−11.40	32.81	0.55	0.45(NS) (0/10)
	Symmetric extinction (μ_0—μ_1)	5	1.22	4.40	2.58×10^{-8}		2.54	1.74	−11.13	32.25	0.00	1.00(NS) (0/10)
	Symmetric transition rate (q_1—q_{10})	5	1.65	4.30	1.75	5.56×10^{-9}	1.89		−11.19	32.38	0.12	0.72(NS) (0/10)

Parameters: λ, speciation rate; μ, extinction rate; q, character transition rate.

Character states: A-0 autogamous; A-1 xenogamous; B-0 annual; B-1 perennial; C-0 small wingless seeds; C-1 large winged seeds; D-0 wide variety of substrates; D-1-specific substrates.

Number of parameters (d.f.), Ln-likelihood (lnLik), Akaike information criterion (AIC) and log-likelihood ratio test (Pr) are provided for comparison between models. Significance codes: (NS) not significant; *≤0.05.

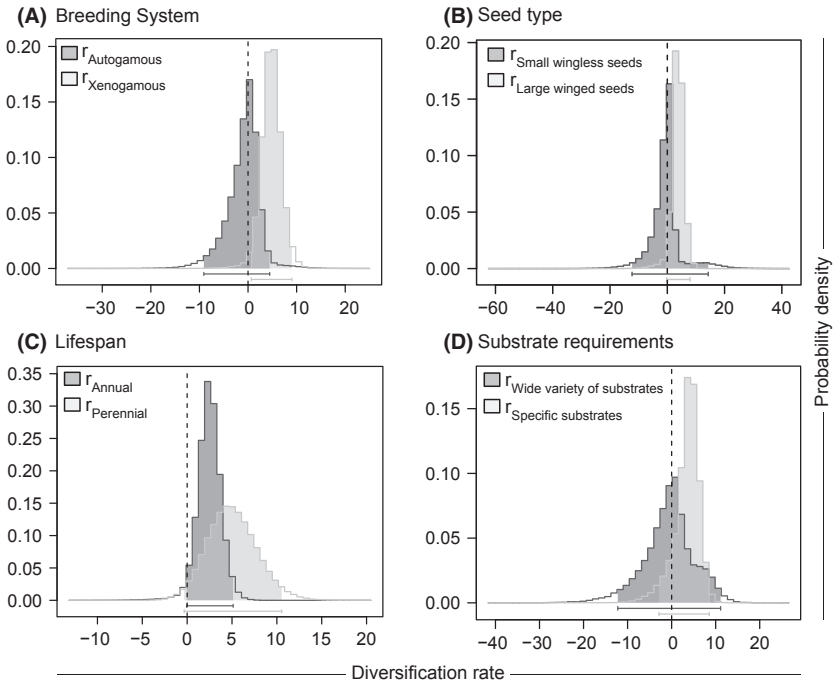

Fig. 5 Results of the binary-state speciation and extinction MCMC-BiSSE analyses performed in ten *Supinae* species trees randomly chosen from the posterior distribution of the *BEAST analysis. It is shown the posterior distribution of diversification rate associated with: (A) breeding system, (B) seed type, (C) lifespan and (D) substrate requirements. Horizontal bars below distributions indicate the 95% credibility interval.

Traits associated with colonization success

Species of the *ssArv* lineage established new populations after LDD of winged seeds (Fig. 4A). However, this morphological trait does not appear to have been a determinant of range expansion success in *Supinae*. SIMMAP analysis of character associations did not support a significant relationship between seed dispersal structures and range expansion ability (Table 2). This is not surprising because, as documented before, the relationship between morphologically defined dispersal syndromes and LDD is weak (Higgins *et al.* 2003; Vargas *et al.* 2012). Specifically in *Linaria*, it has been observed that the wing of the seed is not a successful LDD structure because winged seeds are mainly deposited at short distances from the mother plant (>80% within <0.5 m; Nadeau & King 1991). Furthermore, LDD events over the Mediterranean Sea have been described in *Linaria* sect. *Versicolores*, which possess seeds without wings (Fernández-Mazuecos & Vargas 2011).

By contrast, the SIMMAP correlation analyses detected a positive association between range expansion and the ability to tolerate a wide variety of substrates (Table 2). This suggests that one factor determining the range expansion of *Supinae* species was their ability to become established in areas with different substrates (including in disturbed landscapes). After dispersal, the successful establishment of Mediterranean plants in a new habitat is dependent on suitable climatic and ecological conditions, the major constraints on plant survival (Rodríguez-Sánchez *et al.* 2008; Fernández-Mazuecos & Vargas 2010). Species of the *ssArv* lineage are weeds usually found in pastures, crops and field borders, and thus a possible explanation for their distribution is recent range expansion associated with human agricultural movements, which started *c.* 7500 years ago in the western Mediterranean, (Zeder 2008; Arrigo *et al.* 2010). Dispersal of *ssArv* species is estimated to have occurred throughout the last 0.2 Ma (median age of *ssArv* MRCA; Fig. 3A), at a time that include human perturbations. However, it is unlikely that human-mediated LDD was solely responsible for the spread of *ssArv* species. This is supported by the fact that six *ssSup* species (*L. amethystea*, *L. bipunctata*, *L. caesia*, *L. diffusa*, *L. oligantha* and *L. supina*) are also associated with pastures, crops and field borders (as are *ssArv* species), but their ranges are narrow (see Fig. 1), which leads us to suggest that other traits may also be co-responsible for such contrasting distributions.

Another trait significantly correlated with range expansion was lifespan (Table 2). Despite the association found here, other studies have shown that lifespan

Table 4 Parameter values of the MCMC-BiSSE analyses performed in 10 random trees from the stable posterior distribution of *BEAST analysis. Mean (in brackets) and 95% credibility intervals of the parameter values are shown (see also Fig. 5 and Fig. S2, Supporting information)

Character	λ_0	λ_1	μ_0	μ_1	r_0	r_1	q_1	q_{10}
A—Breeding system	0.07–7.69 (2.25)	3.81–12.27 (7.42)	0.08–11.47 (3.25)	0.08–8.55 (2.65)	−9.14–4.42 (−1.00)	0.52–8.89 (4.76)	0.18–14.03 (3.20)	0.55–8.73 (3.41)
B—Lifespan	1.93–7.79 (4.34)	3.75–13.92 (7.90)	0.06–6.13 (1.91)	0.08–9.45 (2.86)	−0.28–4.94 (2.42)	−0.08–10.83 (5.03)	0.10–2.15 (0.72)	0.01–4.09 (0.72)
C—Seed type	0.05–20.58 (3.17)	1.99–10.23 (6.14)	0.07–15.81 (3.68)	0.07–15.81 (3.68)	−11.78–14.87 (−0.51)	−1.07–7.45 (3.63)	0.09–31.21 (4.79)	0.03–4.54 (1.08)
D—Substrate requirements	0.13–12.80 (4.06)	0.66–11.00 (6.14)	0.12–16.25 (4.72)	0.08–8.63 (2.74)	−13.62–10.10 (−0.65)	−4.04–7.76 (3.40)	0.30–19.38 (6.37)	0.66–9.27 (3.60)

Parameters: λ, speciation rate; μ, extinction rate; r, diversification rate; q, character transition rate.
Character states: A-0 autogamous; A-1 xenogamous; B-0 annual; B-1 perennial; C-0 small wingless seeds; C-1 large winged seeds; D-0 wide variety of substrates; D-1 specific substrates.

is a poor predictor of plant invasiveness (Lonsdale 1994; Goodwin *et al.* 1999; Kolar & Lodge 2001). Nevertheless, it has been predicted that a short lifespan may confer a competitive advantage during range expansions when it is associated with selfing (Sutherland 2004), and both characters are strongly associated in *Supinae* as indicated by our full SIMMAP analysis (Table S7, Supporting information).

The breeding system was also correlated with range expansion in the current study (Table 2). Self-compatibility has been historically proposed as a key trait that facilitates establishment after LDD (Baker's rule; Baker 1955). However, the effect of self-compatibility on the establishment of new populations is still under debate (see references in Table 1). In contrast to Baker's rule, several authors have suggested that self-incompatibility may promote establishment by maximizing variation in the progeny (Carlquist 1966; Lowry & Lester 2006). Nevertheless, recent metapopulation models have indicated that selection for self-compatibility intensifies when a population is newly formed by a low number of immigrants (Pannell & Barrett 1998; Dornier *et al.* 2008), as may have occurred if *ssArv* populations became established following stochastic LDD events. Recent studies have highlighted the correlation between self-compatibility and range expansion success in several groups of angiosperms (Schueller 2004; Busch 2005), including other Plantaginaceae (Randle *et al.* 2009). Self-compatible colonizers have a guaranteed ability to reproduce, in contrasts to outcrossers that require specific pollinators and may experience limited availability of pollen (Ashman *et al.* 2004). In *Supinae*, this is supported: species-specific pollinators (long-tongued bees, such as *Xylocopa*, *Antophora, Rhodanthidium* and *Chalicodoma*) were found to be required for the reproduction of ten narrow-ranged *ssSup* species (Sánchez-Lafuente 2007; Sánchez-Lafuente *et al.* 2012; Blanco-Pastor JL, Romero D, Liberal I, Gómez JM, Ornosa C, Vargas P, unpublished). By contrast, there is no evidence for pollination of *ssArv* species (Knuth 1909; Blanco-Pastor *et al.*, unpublished). In summary, our results indicate that a combination of the three traits that are significantly associated with range expansion (reproductive assurance, short generation time and the ability to tolerate a wide variety of substrates, see Table 2) may have led to the highest degree of range expansion success within the study group, as shown by the wide Mediterranean distribution of the three *ssArv* species (see Fig. 1).

Traits associated with diversification success

The results of the BiSSE analyses showed that the net diversification rate may be linked with breeding system, as evidenced by the contrasting values for

xenogamy-associated diversification and autogamy-associated diversification (Table 4, Fig. 5). In our analyses, contrasting diversification rates are explained by unequal speciation rates, which are higher for xenogamous species (Table 3). This result contrasts with recent research indicating that selfing may promote between-population differentiation (Hamrick & Godt 1996; Charlesworth & Pannell 2001; see also Segarra-Moragues & Mateu-Andres 2007 for an example with some *Linaria* species) and speciation (Wendt *et al.* 2002; Martin & Willis 2007; Guo *et al.* 2009; Goldberg *et al.* 2010). Our results support the view of classical biogeographers: that is, speciation driven by fine adaptation of ecotypes through extensive recombination and heterozygosity via outcrossing. In this view, selfing is associated with quick establishment of genetically constant populations in a wide variety of climates and habitats (Stebbins 1957; Baker 1965) but not with speciation. Nevertheless, differential speciation associated with breeding system was marginally supported in the Bayesian analysis that incorporated uncertainty in the parameter estimates and in the phylogeny (Table 4 and Fig. S2, Supporting information). Also, BiSSE analyses should be interpreted with caution because they achieve very low power when testing rate asymmetry in phylogenies with a low numbers of taxa. In such cases (like the one reported here), a type II error—failing to reject the null hypothesis (e.g. symmetric extinction or character transition rates) when the alternate hypothesis is true—is very likely (Davis *et al.* 2013). In addition, because of the low number of taxa used, we did not check parameter values in a more complex model accounting for a cladogenetic mode of state of change, which may be common in breeding system transitions (Goldberg & Igić 2012). Our results indicate that additional studies are required to quantify the net effect of breeding systems on speciation rates.

Concluding remarks

Linaria sect. *Supinae* species originated in Iberia during the Quaternary and employed two distinct evolutionary strategies. The following autecological traits were found to be responsible for the range expansion success of three weedy species distributed throughout the Mediterranean basin: (i) the ability to tolerate a wide variety of substrates, (ii) a short generation time and (iii) the ability to self-fertilize. This strategy enabled a minority of species to maintain low differentiation and survive in a wide variety of habitats. By contrast, cross-fertilization appears to have promoted specialization (i.e. higher speciation rates) in relatively more specific habitats, leading to a high number of narrow endemics with low colonization ability.

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References

Alvarez N, Thiel-Egenter C, Tribsch A *et al.* (2009) History or ecology? Substrate type as a major driver of patial genetic structure in Alpine plants. *Ecology Letters*, **12**, 632–640.

Angert AL, Crozier LG, Rissler LJ *et al.* (2011) Do species' traits predict recent shifts at expanding range edges? *Ecology Letters*, **14**, 677–689.

Arrigo N, Felber F, Parisod C *et al.* (2010) Origin and expansion of the allotetraploid *Aegilops geniculata*, a wild relative of wheat. *New Phytologist*, **187**, 1170–1180.

Ashman T-L, Knight TM, Steets JA *et al.* (2004) Pollen limitation of plant reproduction: ecological and evolutionary causes and consequences. *Ecology*, **85**, 2408–2421.

Baker H (1955) Self-compatibility and establishment after 'long-distance' dispersal. *Evolution*, **9**, 347–349.

Baker HG (1965) Characteristics and modes of origin of weeds. In: *The Genetics of Colonizing Species* (eds Baker H & Stebbins G), pp. 147–168. Academic Press, New York.

Baker H (1967) Support for Baker's law-as a rule. *Evolution*, **21**, 853–856.

Barrón E, Rivas-Carballo R, Postigo-Mijarra JM *et al.* (2010) The Cenozoic vegetation of the Iberian Peninsula: a synthesis. *Review of Palaeobotany and Palynology*, **162**, 382–402.

Bastida JM, Alcántara JM, Rey PJ, Vargas P, Herrera CM (2010) Extended phylogeny of *Aquilegia*: the biogeographical and ecological patterns of two simultaneous but contrasting radiations. *Plant Systematics and Evolution*, **284**, 171–185.

Bell CD, Mavrodiev E, Soltis P *et al.* (2012) Rapid diversification of *Tragopogon* and ecological associates in Eurasia. *Journal of evolutionary biology*, **25**, 2470–2480.

Blanco-Pastor JL, Vargas P, Pfeil BE (2012) Coalescent simulations reveal hybridization and incomplete lineage sorting in Mediterranean *Linaria*. *PLoS ONE*, **7**, e39089.

Blanco-Pastor JL, Fernández-Mazuecos M, Vargas P (2013) Past and future demographic dynamics of alpine species: limited genetic consequences despite dramatic range contraction in a plant from the Spanish Sierra Nevada. *Molecular Ecology*, **22**, 4177–4195.

Bollback JP (2006) SIMMAP: stochastic character mapping of discrete traits on phylogenies. *BMC Bioinformatics*, **7**, 88

Brown J (1996) The geographic range: size, shape, boundaries, and internal structure. *Annual Review of Ecology and Systematics*, **27**, 597–623.

Busch JW (2005) The evolution of self-compatibility in geographically peripheral populations of *Leavenworthia alabamica* (Brassicaceae). *American Journal of Botany*, **92**, 1503–1512.

Cadotte M, Murray B, Lovett-Doust J (2006) Ecological patterns and biological invasions: using regional species inventories in macroecology. *Biological Invasions*, **8**, 809–821.

Caley P, Groves RH, Barker R (2008) Estimating the invasion success of introduced plants. *Diversity and Distributions*, **14**, 196–203.

Carlquist S (1966) The biota of long-distance dispersal. IV. Genetic systems in the floras of Oceanic Islands. *Evolution*, **20**, 433–455.

Charlesworth D, Pannell J (2001) Mating systems and population genetic structure in the light of coalescent theory. *British Ecological Society*, **14**, 73–96.

Clement M, Posada D, Crandall KA (2000) TCS: a computer progam to estimate gene genealogies. *Molecular Ecology*, **9**, 1657–1660.

Corriveau JL, Coleman AW (1988) Rapid screening method to detect potential biparental inheritance of plastid DNA and results for over 200 angiosperm species. *American Journal of Botany*, **75**, 1443–1458.

Davis M, Midford P, Maddison W (2013) Exploring power and parameter estimation of the BiSSE method for analyzing species diversification. *BMC Evolutionary Biology*, **13**, 38.

Desamore A, Laenen B, Devos N *et al.* (2011) Out of Africa: north-westwards Pleistocene expansions of the heather *Erica arborea*. *Journal of Biogeography*, **38**, 164–176.

Dornier A, Munoz F, Cheptou P-O (2008) Allee effect and self-fertilization in hermaphrodites: reproductive assurance in a structured metapopulation. *Evolution*, **62**, 2558–2569.

Drummond CS (2008) Diversification of *Lupinus* (Leguminosae) in the western New World: derived evolution of perennial life history and colonization of montane habitats. *Molecular Phylogenetics and Evolution*, **48**, 408–421.

Dullinger S, Willner W, Plutzar C *et al.* (2012) Post-glacial migration lag restricts range filling of plants in the European Alps. *Global Ecology and Biogeography*, **21**, 829–840.

Elisens WJ, Tomb AS (1983) Seed morphology in New World Antirrhineae (Scrophulariaceae): systematic and phylogenetic implications. *Plant Systematics and Evolution*, **142**, 23–47.

Escudero M, Vargas P, Arens P, Ouborg NJ, Luceño M (2010) The east-west-north colonization history of the Mediterranean and Europe by the coastal plant *Carex extensa* (Cyperaceae). *Molecular Ecology*, **19**, 352–370.

Fernández-Mazuecos M, Vargas P (2010) Ecological rather than geographical isolation dominates Quaternary formation of Mediterranean *Cistus* species. *Molecular Ecology*, **19**, 1381–1395.

Fernández-Mazuecos M, Vargas P (2011) Historical isolation versus recent long-distance connections between Europe and Africa in bifid toadflaxes (*Linaria* sect. *Versicolores*). *PLoS ONE*, **6**, e22234

Fernández-Mazuecos M, Blanco-Pastor JL, Vargas P (2013) A phylogeny of toadflaxes (*Linaria* Mill.) based on nuclear internal transcribed spacer sequences: systematic and evolutionary consequences. *International Journal of Plant Sciences*, **174**, 234–249.

Ferrer MM, Good SV (2012) Self-sterility in flowering plants: preventing self-fertilization increases family diversification rates. *Annals of Botany*, **110**, 535–553.

Fitzjohn RG, Maddison WP, Otto SP (2009) Estimating trait-dependent speciation and extinction rates from incompletely resolved phylogenies. *Systematic Biology*, **58**, 595–611.

Fiz-Palacios O, Valcárcel V (2013) From Messinian crisis to Mediterranean climate: a temporal gap of diversification recovered from multiple plant phylogenies. *Perspectives in Plant Ecology, Evolution and Systematics*, **15**, 130–137.

Fiz-Palacios O, Vargas P, Vila R, Papadopulos AST, Aldasoro JJ (2010) The uneven phylogeny and biogeography of *Erodium* (Geraniaceae): radiations in the Mediterranean and recent recurrent intercontinental colonization. *Annals of Botany*, **106**, 871–884.

Gassó N, Sol D, Pino J *et al.* (2009) Exploring species attributes and site characteristics to assess plant invasions in Spain. *Diversity and Distributions*, **15**, 50–58.

Goldberg EE, Igić B (2012) Tempo and mode in plant breeding system evolution. *Evolution*, **66**, 3701–3709.

Goldberg EE, Kohn JR, Lande R *et al.* (2010) Species selection maintains self-incompatibility. *Science*, **330**, 493–495.

Goodwin BJ, McAllister AJ, Fahrig L (1999) Predicting invasiveness of plant species based on biological information. *Conservation Biology*, **13**, 422–426.

Guo YL, Bechsgaard JS, Slotte T *et al.* (2009) Recent speciation of *Capsella rubella* from *Capsella grandiflora*, associated with loss of self-incompatibility and an extreme bottleneck. *Proceedings of the National Academy of Sciences*, **106**, 5246–5251.

Guzmán B, Vargas P (2009) Long-distance colonization of the Western Mediterranean by *Cistus ladanifer* (Cistaceae) despite the absence of special dispersal mechanisms. *Journal of Biogeography*, **36**, 954–968.

Guzmán B, Lledó MD, Vargas P (2009) Adaptive radiation in mediterranean *Cistus* (Cistaceae). *PLoS ONE*, **4**, e6362.

Hamilton MB (1999) Four primer pairs for the amplification of chloroplast intergenic regions with intraspecific variation. *Molecular Ecology*, **8**, 521–523.

Hamrick JL, Godt MJW (1996) Effects of life history traits on genetic diversity in plant species. *Philosophical Transactions: Biological Sciences*, **351**, 1291–1298.

Harris AJ, Xiang QY (2009) Estimating ancestral distributions of lineages with uncertain sister groups: a statistical approach to dispersal-vicariance analysis and a case using *Aesculus* L. (Sapindaceae) including fossils. *Journal of Systematics and Evolution*, **47**, 349–368.

Heled J, Drummond AJ (2010) Bayesian inference of species trees from multilocus data. *Molecular Biology and Evolution*, **27**, 570–580.

Higgins SI, Nathan R, Cain ML (2003) Are long-distance dispersal events in plants usually caused by nonstandard means of dispersal? *Ecology*, **84**, 1945–1956.

Huelsenbeck JP, Nielsen R, Bollback JP (2003) Stochastic mapping of morphological characters. *Systematic Biology*, **52**, 131–158.

Jiménez-Moreno G, Fauquette S, Suc J-P (2010) Miocene to Pliocene vegetation reconstruction and climate estimates in the Iberian Peninsula from pollen data. *Review of Palaeobotany and Palynology*, **162**, 403–415.

Kadereit JW, Griebeler EM, Comes HP (2004) Quaternary diversification in European alpine plants: pattern and process. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, **359**, 265–274.

van Kleunen M, Johnson SD (2007) Effects of self-compatibility on the distribution range of invasive European plants in North America. *Conservation Biology*, **21**, 1537–1544.

Knuth P (1909) *Handbook of Flower Pollination: Based Upon Hermann Müller's Work 'The Fertilisation of Flowers by Insects'*. Clarendon Press, Oxford.

Kolar CS, Lodge DM (2001) Progress in invasion biology: predicting invaders. *Trends in Ecology & Evolution*, **16**, 199–204.

Lake JC, Leishman MR (2004) Invasion success of exotic plants in natural ecosystems: the role of disturbance, plant attributes and freedom from herbivores. *Biological Conservation*, **117**, 215–226.

Leaché AD, Harris RB, Rannala B, Yang Z (2013) The influence of gene flow on species tree estimation: a simulation study. *Systematic Biology*. doi: 10.1093/sysbio/syt049.

Lewis PO (2001) A likelihood approach to estimating phylogeny from discrete morphological character data. *Systematic Biology*, **50**, 913.

Liepman AH, Olsen LJ (2001) Peroxisomal alanine : glyoxylate aminotransferase (AGT1) is a photorespiratory enzyme with multiple substrates in *Arabidopsis thaliana*. *The Plant Journal*, **25**, 487–498.

Liu L, Yu L, Kubatko L, Pearl DK, Edwards SV (2009) Coalescent methods for estimating phylogenetic trees. *Molecular Phylogenetics and Evolution*, **53**, 320–328.

Lo Presti RM, Oberprieler C (2009) Evolutionary history, biogeography and eco-climatological differentiation of the genus *Anthemis* L.(Compositae, Anthemideae) in the circum-Mediterranean area. *Journal of Biogeography*, **36**, 1313–1332.

Lo Presti RM, Oberprieler C (2011) The central Mediterranean as a phytodiversity hotchpotch: phylogeographical patterns of the *Anthemis secundiramea* group (Compositae, Anthemideae) across the Sicilian Channel. *Journal of Biogeography*, **38**, 1109–1124.

Lonsdale WM (1994) Inviting trouble: introduced pasture species in northern Australia. *Australian Journal of Ecology*, **19**, 345–354.

Lowry E, Lester SE (2006) The biogeography of plant reproduction: potential determinants of species' range sizes. *Journal of Biogeography*, **33**, 1975–1982.

Lyford ME, Jackson ST, Betancourt JL, Gray ST (2003) Influence of landscape structure and climate variability on a late holocene plant migration. *Ecological Monographs*, **73**, 567–583.

Maddison WP, Maddison DR (2009) *Mesquite: A Modular System for Evolutionary Analysis*. Version 2.6. Available at: http: mesquiteproject.org.

Maddison WP, Midford PE, Otto SP (2007) Estimating a binary character's effect on speciation and extinction. *Systematic Biology*, **56**, 701–710.

Martin NH, Willis JH (2007) Ecological divergence associated with mating system causes nearly complete reproductive isolation between sympatric *Mimulus* species. *Evolution*, **61**, 68–82.

Martín-Bravo S, Valcárcel V, Vargas P, Luceño M (2010) Geographical speciation related to Pleistocene range shifts in the western mediterranean mountains (*Reseda* sect. *Glaucoreseda*, *Resedaceae*). *Taxon*, **59**, 466–482.

Nadeau LB, King JR (1991) Seed dispersal and seedling establishment of *Linaria vulgaris* Mill. *Canadian Journal of Plant Science*, **71**, 771–782.

Nylander JA, Olsson U, Alström P, Sanmartín I (2008) Accounting for phylogenetic uncertainty in biogeography: a Bayesian approach to dispersal-vicariance analysis of the thrushes (Aves: *Turdus*). *Systematic Biology*, **57**, 257–268.

Palamarev E (1989) Paleobotanical evidences of the Tertiary history and origin of the Mediterranean sclerophyll dendroflora. *Plant Systematics and Evolution*, **162**, 93–107.

Pannell JR, Barrett SCH (1998) Baker's law revisited: reproductive assurance in a metapopulation. *Evolution*, **52**, 657–668.

Petit RJ, Hampe A (2006) Some evolutionary consequences of being a tree. *Annual Review of Ecology, Evolution, and Systematics*, **37**, 187–214.

Postigo-Mijarra JM, Morla C, Barrón E, Morales-Molino C, García S (2010) Patterns of extinction and persistence of Arcto-tertiary flora in Iberia during the Quaternary. *Review of Palaeobotany and Palynology*, **162**, 416–426.

Pyšek P, Sádlo J, Mandák B, Jarošík V (2003) Czech alien flora and the historical pattern of its formation: what came first to Central Europe? *Oecologia*, **135**, 122–130.

Rambuda TD, Johnson SD (2004) Breeding systems of invasive alien plants in South Africa: does Baker's rule apply? *Diversity and Distributions*, **10**, 409–416.

Randle AM, Slyder JB, Kalisz S (2009) Can differences in autonomous selfing ability explain differences in range size among sister-taxa pairs of *Collinsia* (Plantaginaceae)? An extension of Baker's Law. *New Phytologist*, **183**, 618–629.

Reichard SH, Hamilton CW (1997) Predicting invasions of woody plants introduced into North America. *Conservation Biology*, **11**, 193–203.

Rodger JG, van Kleunen M, Johnson SD (2013) Pollinators, mates and Allee effects: the importance of self-pollination for fecundity in an invasive lily. *Functional Ecology*, **27**, 1023–1033.

Rodríguez-Sánchez F, Pérez-Barrales R, Ojeda F, Vargas P, Arroyo J (2008) The Strait of Gibraltar as a melting pot for plant biodiversity. *Quaternary Science Reviews*, **27**, 2100–2117.

Ronquist F (1997) Dispersal-vicariance analysis: a new approach to the quantification of historical biogeography. *Systematic Biology*, **46**, 195–203.

Rouy G (1883) Matériaux pour servir à la révision de la flore portugaise accompagnés de notes sur certaines espèces ou variétés critiques de plantes européennes. *Le Naturaliste*, **5**, 349–351.

Sáez L, Bernal M (2009) *Linaria* Mill. In: *Flora Iberica 13* (eds Castroviejo S, Herrero A, Benedí C, Rico E & Güemes J), pp. 232–324. CSIC, Madrid, Spain.

Sánchez-Lafuente A (2007) Corolla herbivory, pollination success and fruit predation in complex flowers: an experimental study with *Linaria lilacina* (Scrophulariaceae). *Annals of Botany*, **99**, 355–364.

Sánchez-Lafuente A, Rodríguez-Gironés M, Parra R (2012) Interaction frequency and per-interaction effects as predictors of total effects in plant–pollinator mutualisms: a case study with the self-incompatible herb *Linaria lilacina*. *Oecologia*, **168**, 153–165.

Schaal BA, Hayworth DA, Olsen KM, Rauscher JT, Smith WA (1998) Phylogeographic studies in plants: problems and prospects. *Molecular Ecology*, **7**, 465–474.

Schueller SK (2004) Self-pollination in island and mainland populations of the introduced hummingbird-pollinated plant, *Nicotiana glauca* (Solanaceae). *American Journal of Botany*, **91**, 672–681.

Segarra-Moragues JG, Mateu-Andres I (2007) Levels of allozyme diversity in closely related toadflaxes (*Linaria*, Plantaginaceae) and their correspondence with the breeding systems of the species. *Conservation Genetics*, **8**, 373–383.

Shaw J, Lickey EB, Schilling EE, Small RL (2007) Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: the Tortoise and the hare III. *American Journal of Botany*, **94**, 275–288.

Smith SA, Beaulieu JM (2009) Life history influences rates of climatic niche evolution in flowering plants. *Proceedings of the Royal Society B: Biological Sciences*, **276**, 4345–4352.

Smith SA, Donoghue MJ (2008) Rates of molecular evolution are linked to life history in flowering plants. *Science*, **322**, 86–89.

Stebbins GL (1950) *Variation and Evolution in Plants*. Columbia University Press, New York.

Stebbins GL (1957) Self fertilization and population variability in the higher plants. *The American Naturalist*, **91**, 337–354.

Stout JC (2007) Reproductive biology of the invasive exotic shrub, *Rhododendron ponticum* L. (Ericaceae). *Botanical Journal of the Linnean Society*, **155**, 373–381.

Suc JP (1984) Origin and evolution of the Mediterranean vegetation and climate in Europe. *Nature*, **307**, 429–432.

Sutherland S (2004) What makes a weed a weed: life history traits of native and exotic plants in the USA. *Oecologia*, **141**, 24–39.

Sutton DA (1988) *A Revision of the Tribe Antirrhineae*. Oxford University Press, London, UK.

Taberlet P, Gielly L, Pautou G, Bouvet J (1991) Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology*, **17**, 1105–1109.

Thompson JD (2005) *Plant Evolution in the Mediterranean*. Oxford University Press, New York.

Thompson K, Gaston KJ, Band SR (1999) Range size, dispersal and niche breadth in the herbaceous flora of central England. *Journal of Ecology*, **87**, 150–155.

Valdés B (1970a) *Revisión de las especies europeas de Linaria con semillas aladas*. Anales de la Universidad Hispalense, Sevilla, Spain.

Valdés B (1970b) Taxonomía experimental del género *Linaria* IV. Reproducción sexual: autogamia y polinización intraespecífica. *Boletín de la Real Sociedad Española de Historia Natural (Biológica)*, **68**, 79–89.

Valdés B, Cabezudo B (1977) Linaria tursica Valdés & Cabezudo, sp. nov. *Lagascalia*, **7**, 9–12.

Valente LM, Vargas P (2013) Contrasting evolutionary hypotheses between two mediterranean-climate floristic hotspots: the Cape of southern Africa and the Mediterranean Basin. *Journal of Biogeography*, doi: 10.1111/jbi.12156.

Valente LM, Savolainen V, Vargas P (2010) Unparalleled rates of species diversification in Europe. *Proceedings of the Royal Society B: Biological Sciences*, **277**, 1489–1496.

Vargas P, Carrió E, Guzmán B, Amat E, Güemes J (2009) A geographical pattern of *Antirrhinum* (Scrophulariaceae) speciation since the Pliocene based on plastid and nuclear DNA polymorphism. *Journal of Biogeography*, **36**, 1297–1312.

Vargas P, Heleno R, Traveset A, Nogales M (2012) Colonization of the Galápagos Islands by plants with no specific syndromes for long-distance dispersal: a new perspective. *Ecography*, **35**, 33–43.

van der Veken S, Bellemare J, Verheyen K, Hermy M (2007) Life-history traits are correlated with geographical distribution patterns of western European forest herb species. *Journal of Biogeography*, **34**, 1723–1735.

Verboom GA, Linder HP, Stock WD (2004) Testing the adaptive nature of radiation: growth form and life history divergence in the African grass genus *Ehrharta* (Poaceae: Ehrhartoideae). *American Journal of Botany*, **91**, 1364–1370.

Wendt T, Canela MBF, Klein DE, Rios RI (2002) Selfing facilitates reproductive isolation among three sympatric species of *Pitcairnia* (Bromeliaceae). *Plant Systematics and Evolution*, **232**, 201–212.

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols. A Guide to Methods and Applications* (eds. Innis MA, Gelfond DH, Sninsky JJ, White TJ), vol. 18, pp. 315–322. Academic Press, San Diego.

Willis KJ, Niklas KJ (2004) The role of Quaternary environmental change in plant macroevolution: the exception or the rule? *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, **359**, 159–172.

Wilson EO (1965) The challenge from related species. In: *The Genetics of Colonizing Species* (eds Baker HG & Stebbins GL), pp. 7–24. Academic Press New York, New York.

Yu Y, Harris AJ, He X (2010) S-DIVA (Statistical Dispersal-Vicariance Analysis): a tool for inferring biogeographic histories. *Molecular Phylogenetics and Evolution*, **56**, 848–850.

Yu Y, Harris AJ, He X-J (2011) *RASP (Reconstruct Ancestral State in Phylogenies) 2.0 beta*. Available at http://mnh.scu.edu.cn/soft/blog/RASP.

Zeder MA (2008) Domestication and early agriculture in the Mediterranean Basin: origins, diffusion, and impact. *Proceedings of the National Academy of Sciences of the United States of America*, **105**, 11597–11604.

J.L.B. designed research, performed research, analysed data, wrote the paper. P.V. designed research, wrote the paper. Both authors contributed to data interpretation and proofreading.

Data accessibility

Sample information including GenBank accession numbers, sequence alignments, breeding system experiment data and states of character data: Supporting information.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Phylogenetic relationships of *Linaria* plastid DNA accessions.

Fig. S2 Results of the binary-state speciation and extinction

MCMC-BiSSE analyses. It is shown the posterior distribution of speciation, extinction and character transition rates.

Table S1 The 120 samples of *Linaria* sect. *Supinae* and out-group material used for sequencing.

Table S2 Characteristics of the DNA regions sequenced for the present study.

Table S3 Plastid haplotype numbers of *Linaria* sect. *Supinae* samples as shown in Fig. 4.

Table S4 Breeding system data.

Table S5 Plants included in the breeding system tests of the present study with localities and detailed measures of the pollen/ovule ratio tests.

Table S6 States of characters as used in: (i) SIMMAP, (ii) Mesquite, and (iii) BiSSE analyses.

Appendix S1 Supplementary Materials and Methods.

Data S1 Sequence alignment input files used in the present study: (a) *rpl*32-*trn*L^{UAG}, (b) *trn*S-*trn*G, (c) *trn*L-*trn*F, (d) ITS and (e) AGT1.

Supporting Information from Blanco-Pastor & Vargas 2013, "Autecological traits determined two evolutionary strategies in Mediterranean Plants during the Quaternary: low differentiation and range expansion versus geographical speciation in *Linaria*"

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Supplementary Figures

Fig. S1. Phylogenetic relationships of *Linaria* plastid DNA accessions (concatenated *rpl32-trnL*^{UAG}, *trnL-trnF* and *trnS-trnG* regions) based on the 50% majority-rule consensus tree of the Bayesian Inference analysis of MrBayes (Ronquist & Huelsenbeck 2003). Numbers above branches are Bayesian Posterior Probability, number below branches are Maximum Parsimony bootstrap support values as obtained with TNT (Goloboff *et al.* 2008) and Maximum Likelihood bootstrap support values as obtained with PhyML (Guindon *et al.* 2010). See Appendix S1 for further analyses details.

Fig S2. Results of the binary-state speciation and extinction MCMC-BiSSE analyses of ten species trees randomly chosen from the poseterior distribution of the *BEAST analysis. It is shown the posterior distribution of speciation, extinction and character transition rates associated with: (A) breeding system, (B) seed type, (C) lifespan and (D) substrate requirements. Horizontal bars indicate the 95% credibility interval.

Fig. S1

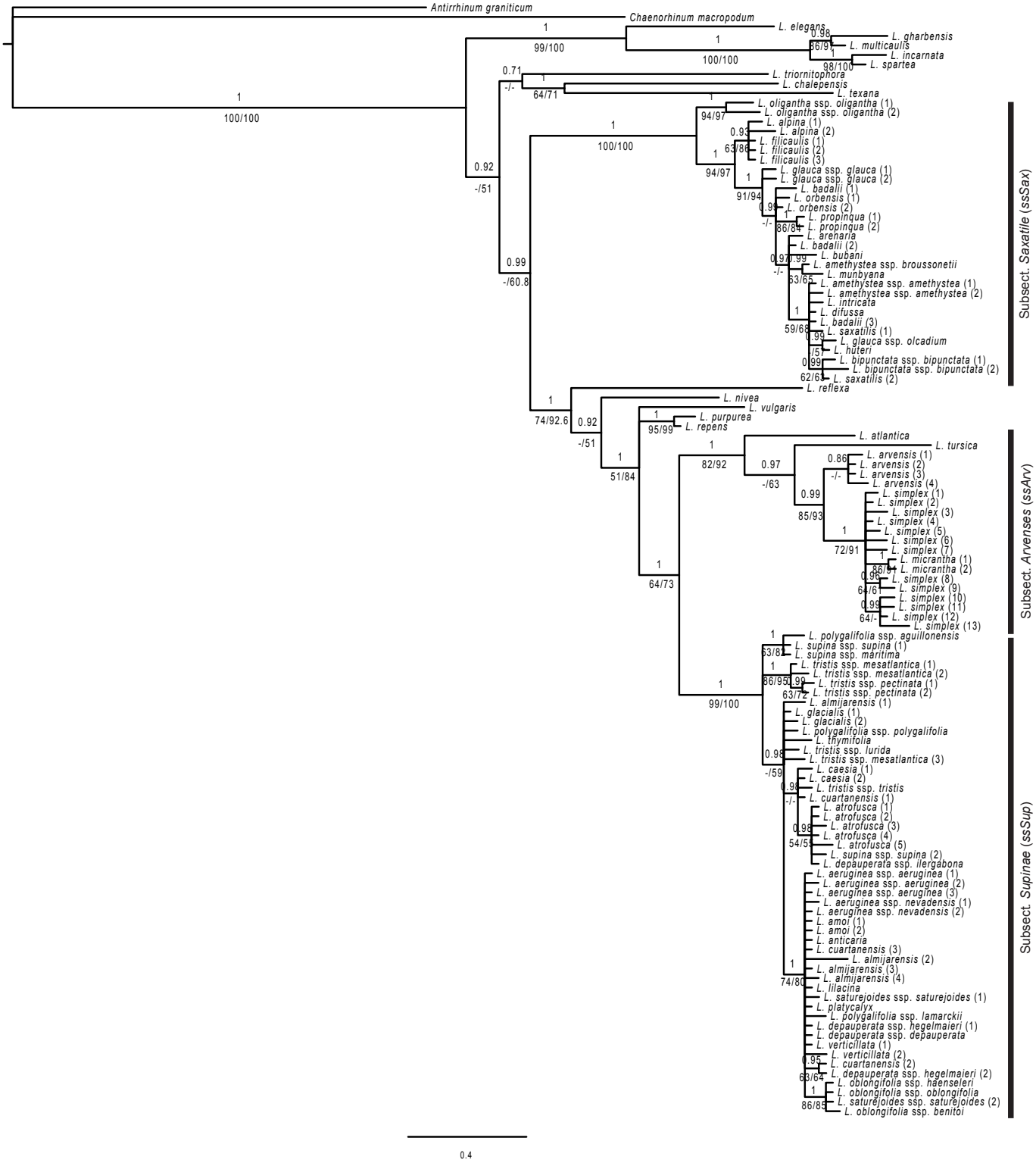
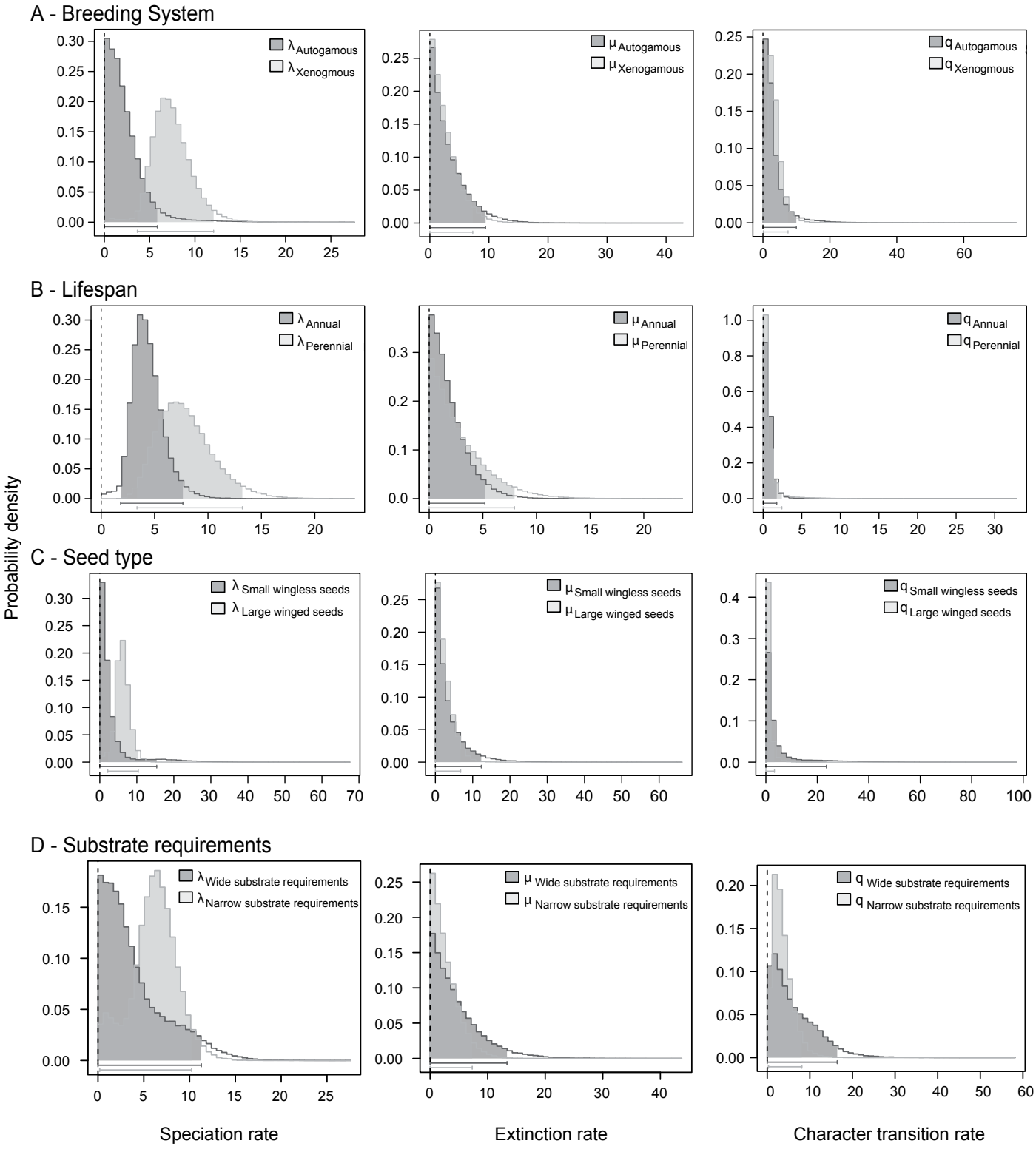

Antirrhinum graniticum
Chaenorhinum macropodum
L. elegans
L. gharbensis
L. multicaulis
L. incarnata
L. spartea
L. triornitophora
L. chalepensis
L. texana
L. oligantha ssp. oligantha (1)
L. oligantha ssp. oligantha (2)
L. alpina (1)
L. alpina (2)
L. filicaulis (1)
L. filicaulis (2)
L. filicaulis (3)
L. glauca ssp. glauca (1)
L. glauca ssp. glauca (2)
L. badalii (1)
L. orbensis (1)
L. orbensis (2)
L. propinqua (1)
L. propinqua (2)
L. arenaria
L. badalii (2)
L. bubani
L. amethystea ssp. broussonetii
L. munbyana
L. amethystea ssp. amethystea (1)
L. amethystea ssp. amethystea (2)
L. intricata
L. difussa
L. badalii (3)
L. saxatilis (1)
L. glauca ssp. olcadium
L. huteri
L. bipunctata ssp. bipunctata (1)
L. bipunctata ssp. bipunctata (2)
L. saxatilis (2)
L. reflexa
L. nivea
L. vulgaris
L. purpurea
L. repens
L. atlantica
L. tursica
L. arvensis (1)
L. arvensis (2)
L. arvensis (3)
L. arvensis (4)
L. simplex (1)
L. simplex (2)
L. simplex (3)
L. simplex (4)
L. simplex (5)
L. simplex (6)
L. simplex (7)
L. micrantha (1)
L. micrantha (2)
L. simplex (8)
L. simplex (9)
L. simplex (10)
L. simplex (11)
L. simplex (12)
L. simplex (13)
L. polygalifolia ssp. aguillonensis
L. supina ssp. supina (1)
L. supina ssp. maritima
L. tristis ssp. mesatlantica (1)
L. tristis ssp. mesatlantica (2)
L. tristis ssp. pectinata (1)
L. tristis ssp. pectinata (2)
L. almijarensis (1)
L. glacialis (1)
L. glacialis (2)
L. polygalifolia ssp. polygalifolia
L. thymifolia
L. tristis ssp. lurida
L. tristis ssp. mesatlantica (3)
L. caesia (1)
L. caesia (2)
L. tristis ssp. tristis
L. cuartanensis (1)
L. atrofusca (1)
L. atrofusca (2)
L. atrofusca (3)
L. atrofusca (4)
L. atrofusca (5)
L. supina ssp. supina (2)
L. depauperata ssp. ilergabona
L. aeruginea ssp. aeruginea (1)
L. aeruginea ssp. aeruginea (2)
L. aeruginea ssp. aeruginea (3)
L. aeruginea ssp. nevadensis (1)
L. aeruginea ssp. nevadensis (2)
L. amoi (1)
L. amoi (2)
L. anticaria
L. cuartanensis (3)
L. almijarensis (2)
L. almijarensis (3)
L. almijarensis (4)
L. lilacina
L. saturejoides ssp. saturejoides (1)
L. platycalyx
L. polygalifolia ssp. lamarckii
L. depauperata ssp. hegelmaieri (1)
L. depauperata ssp. depauperata
L. verticillata (1)
L. verticillata (2)
L. cuartanensis (2)
L. depauperata ssp. hegelmaieri (2)
L. oblongifolia ssp. haenseleri
L. oblongifolia ssp. oblongifolia
L. saturejoides ssp. saturejoides (2)
L. oblongifolia ssp. benitoi
Subsect. Saxatile (ssSax)
Subsect. Arvenses (ssArv)
Subsect. Supinae (ssSup)
0.4

Fig. S2


A - Breeding System
λ Autogamous
λ Xenogmous
μ Autogamous
μ Xenogamous
q Autogamous
q Xenogmous
B - Lifespan
λ Annual
λ Perennial
μ Annual
μ Perennial
q Annual
q Perennial
C - Seed type
λ Small wingless seeds
λ Large winged seeds
μ Small wingless seeds
μ Large winged seeds
q Small wingless seeds
q Large winged seeds
D - Substrate requirements
λ Wide substrate requirements
λ Narrow substrate requirements
μ Wide substrate requirements
μ Narrow substrate requirements
q Wide substrate requirements
q Narrow substrate requirements
Probability density
Speciation rate
Extinction rate
Character transition rate

Supplementary Tables

Table S1. The 120 samples of *Linaria* sect. *Supinae* and outgroup material used for sequencing. Population numbers are given in brackets after species names. Herbarium abbreviations: MA, herbarium of the Royal Botanical Gardens of Madrid (Spain); RNG, herbarium of Reading University (UK); SEV, herbarium of Seville University (Spain). Genbank accession numbers for each DNA region are indicated.

Table S2. Characteristics of the DNA regions sequenced for the present study.

Table S3. Plastid haplotype numbers of *Linaria* sect. *Supinae* samples as shown in Fig. 4.

Table S4. Breeding system data. Number of individuals, pollinated flowers, fruit set and fruit/flower ratio. Range of Pollen/Ovule ratio (P/O) and its breeding system significance following Cruden (1977) is also included.

Table S5. Plants included in the breeding system tests of the present study with localities and detailed measures of the pollen/ovule ratio tests.

Table S6. States of characters used in (i) the SIMMAP species traits association analyses, (ii) the Mesquite ancestral state reconstruction analyses, and (iii) the associated diversification analyses performed under the BiSSE model.

Table S7. Results of the SIMMAP character correlation analysis. It is indicated the character correlation *m* and *d*-statistics among all binary traits analyzed within *Supinae*.

Table S1

	Taxon	Locality	Collector	Collection number	rpl32-trnL^{UAG}	trnS-trnG	trnL-trnF	ITS	AGT1
1	*L. aeruginea* (Gouan) Cav. ssp. *aeruginea* (1)	Spain: Granada. Sierra Nevada. Pradollano	J.L. Blanco Pastor	51JB09	JN663397	JN663616	JN663504	JQ814486	JQ814558
2	*L. aeruginea* (Gouan) Cav. ssp. *aeruginea* (2)	Spain: Jaén. Sierra de Cazorla	M. Fernández-Mazuecos	50MF09	JN663398	JN663617	JN663505	KF623172	KF623212
3	*L. aeruginea* (Gouan) Cav. ssp. *aeruginea* (3)	Spain: Albacete. Yeste	P.F. Cannon et al.	2009.12.80 (RNG)	JN663399	JN663618	JN663506	KF623173	-
4	*L. aeruginea* ssp. *nevadensis* (Boiss.) D.A. Sutton (1)	Spain: Almería. Sierra Nevada. Abrucena	B. Valdés et al.	2009.12.54 (RNG)	JN663400	JN663619	JN663507	KF623174	-
5	*L. aeruginea* ssp. *nevadensis* (Boiss.) D.A. Sutton (2)	Spain: Granada. Sierra Nevada. Pico del Veleta	J.L. Blanco Pastor	44JB09	JN663401	JN663620	JN663508	JQ814487	JQ814559
6	*L. almijarensis* Campo & Amo. (1)	Spain: Cordoba. Sierra Gallinera	J.L. Blanco Pastor	57JB10	JN663410	JN663630	JN663518	KF623175	-
7	*L. almijarensis* Campo & Amo. (2)	Spain: Córdoba. Priego de Cordoba. Las Angosturas	J. Devesa & J. Muñoz	2009.12.18 (RNG)	JN663411	JN663631	JN663519	KF623176	-
8	*L. almijarensis* Campo & Amo. (3)	Spain: Córdoba. Cabra.	J.L. Blanco Pastor	36JB10	JN663412	JN663632	JN663520	JQ814492	JQ814564
9	*L. almijarensis* Campo & Amo. (4)	Spain: Sevilla. Algámitas	P.F. Cannon	2009.12.32 (RNG)	JN663413	JN663633	JN663521	KF623177	-
10	*L. alpina* (L.) Mill. (1)	Slovenia: Karavanke. Zelenica-Palec	B. Frajman	12605BF	JN663402	JN663621	JN663509	KF623178	-
11	*L. alpina* (L.) Mill. (2)	Spain: Huesca. Formigal	S. Martín Bravo	571SMB05	JN663510	JN663622	JN663403	JQ814489	JQ814561
12	*L. amethystea* Hoffmanns. & Link (Vent.) ssp. *amethystea* (1)	Spain: Málaga. Coín	M. Fernández-Mazuecos	26MF09	JN663404	JN663623	JN663511	KF623179	-
13	*L. amethystea* Hoffmanns. & Link (Vent.) ssp. *amethystea* (2)	Spain: Ciudad Real. Ciudad Real	R. García Río	712742 (MA)	JN663405	JN663624	JN663512	JQ814490	JQ814562
14	*L. amethystea* ssp. *broussonetii* (Poiret) Malato-Beliz	Morocco: Ifrane. Ifrane	M. Fernández-Mazuecos	10bisMF08	JN663406	JN663625	JN663513	KF623180	-
15	*L. amoi* Campo ex Amo (1)	Spain: Sierra Tejeda. Canillas de Aceituno	J.L. Blanco Pastor	37JB09	JN663407	JN663626	JN663514	JX481108	KC625806 KC625807
16	*L. amoi* Campo ex Amo (2)	Spain: Málaga. Cómpeta	M. Fernández-Mazuecos & al.	30PV08	JF694122	JN663627	JN663515	KF623181	-
17	*L. anticaria* Boiss. & Reut.	Spain: Málaga. El Torcal de Antequera	J.L. Blanco Pastor	33JB09	JN663408	JN663628	JN663516	JQ814491	JQ814563
18	*L. arenaria* DC.	France. Vendée. Sint Gilles	F. De Raeve	2009.12.174 (RNG)	JN663414	JN663634	JN663522	JX481112	-
19	*L. arvensis* (L.) Desf. (1)	France: Córse. Col de Bigorno	J. Lambinon	2009.12.169 (RNG)	JN663415	JN663635	JN663523	JQ814493	JQ814565
20	*L. arvensis* (L.) Desf. (2)	Spain: Almería. Uleila del Campo	S.L. Jury & R.N. Carter	2009.12.165 (RNG)	JN663416	JN663636	JN663524	JQ814494	JQ814566
21	*L. arvensis* (L.) Desf. (3)	Morocco: Souss-Massa. Daraâ	A. Quintanar & al.	799225 (MA)	JN663417	JN663637	JN663525	-	-
22	*L. arvensis* (L.) Desf. (4)	Spain: Soria. El Royo	C. Aedo & al.	733323 (MA)	JN663418	JN663638	JN663526	-	-
23	*L. atlantica* Boiss & Reut.	Morocco: Kebdana	Sennen & Mauricio	109741 (MA)	JN663419	JN663639	JN663527	-	-

24	*L. atrofusca* (Rouy) (1)	Spain: Ávila. Mengamuñoz	S.L. Jury & R. Morales	2009.12.83 (RNG)	JN663392	JN663611	JN663499	KF623182	-
25	*L. atrofusca* (Rouy) (2)	Spain: Cuenca. El Tobar	M. Fernández-Mazuecos	53MF09	JN663393	JN663612	JN663500	KF623183	-
26	*L. atrofusca* (Rouy) (3)	Spain: Guadalajara. Póveda	P. Jiménez Mejías	35PJM09	JN663394	JN663613	JN663501	-	-
27	*L. atrofusca* (Rouy) (4)	Spain: Madrid. San Lorenzo del Escorial	M.F. & S.G. Gardner	2009.12.96 (RNG)	JN663395	JN663614	JN663502	-	-
28	*L. atrofusca* (Rouy) (5)	Spain: Ciudad Real. Villanueva de la Fuente	T. Luque et al.	2009.12.94 (RNG)	JN663396	JN663615	JN663503	-	-
29	*L. badalii* Loscos (1)	Spain: Huesca. Sopeira	P. Vargas	81PV10	JN663420	JN663640	JN663528	KF623184	-
30	*L. badalii* Loscos (2)	Spain: León. Riaño	M.F. Gardner & S.G. Gardner	2009.12.155 (RNG)	JN663421	JN663641	JN663529	JQ814495	JQ814567
31	*L. badalii* Loscos (3)	Spain: Burgos. Pancorbo	Unknown collector	2009.12.140 (RNG)	JN663460	JN663683	JN663571	-	-
32	*L. bipunctata* (L.) Chaz. ssp. *bipunctata* (1)	Spain: Madrid. Sierra de Guadarrama	Unknown collector	2009.12.11 (RNG)	JN663422	JN663642	JN663530	KF623185	-
33	*L. bipunctata* (L.) Chaz. ssp. *bipunctata* (2)	Spain: Soria. Quintana Redonda	A. Segura	2009.12.7 (RNG)	JN663423	JN663643	JN663531	JQ814496	JQ814568
34	*L. bubanii* Font Quer	Spain: Huesca. El Pueyo de Araguás	M. Carrasco & al.	609430 (MA)	JN663424	JN663644	JN663532	JQ814537 JQ814538	JQ814569
35	*L. caesia* (Pers.) F.G.Dietr. (1)	Spain: Ciudad Real. Daimiel	A. Molina & J. Varela	2009.12.102 (RNG)	JN663425	JN663645	JN663533	JX481105	-
36	*L. caesia* (Pers.) F.G.Dietr. (2)	Spain: Soria. Almazán	A. Segura	2009.12.111 (RNG)	JN663426	JN663646	JN663534	KF623186	-
37	*L. cuartanensis* (Degen & Hervier) Fern. Casas ex. Blanco-Pastor (1)	Spain: Granada. Sierra de Guillimona	B. Valdés et al.	2009.12.21 (RNG)	JN663495	JN663718	JN663606	KF623187	-
38	*L. cuartanensis* (Degen & Hervier) Fern. Casas ex. Blanco-Pastor (2)	Spain: Albacete. Yeste	P. F. Cannon et al.	2009.12.35 (RNG)	JN663444	JN663667	JN663555	JQ814510	JQ814583
39	*L. cuartanensis* (Degen & Hervier) Fern. Casas ex. Blanco-Pastor (3)	Spain: Granada. Sierra de la Sagra	B.Valdés & al.	2009.12.20 (RNG)	JN663409	JN663629	JN663517	KF623188	-
40	*L. depauperata* Leresche ex Lange ssp. *depauperata*	Spain: Alicante. Alcoi	L. Serra	2009.12.157 (RNG)	JN663430	JN663651	JN663539	JQ814499	JQ814572
41	*L. depauperata* ssp. *hegelmaieri* (Lange) De la Torre, Alcaraz & M.B. Crespo (1)	Spain: Alicante. Petrel	J.L. Blanco Pastor	6JB10	JN663429	JN663650	JN663538	-	-
42	*L. depauperata* ssp. *hegelmaieri* (Lange) De la Torre, Alcaraz & M.B. Crespo (2)	Spain: Albacete. Fábrica de Riópar.	P.F. Cannon et al.	2009.12.156 (RNG)	JN663431	JN663652	JN663540	JX481107	-
43	*L. depauperata* ssp. *ilergabona* (M.B. Crespo & Arán) L. Sáez	Spain: Castellón. Vistabella	S.L. Jury et al.	2009.12.85 (RNG)	JN663428	JN663649	JN663537	KF623189	-
44	*L. diffusa* Hoffmanns. & Link.	Portugal: Freixo-de-espada à Cinta	A. Teixeira	s.n.	JN663432	JN663653	JN663541	JX481114	-
45	*L. filicaulis* Boiss. ex Leresche & Levier (1)	Spain: León. Pico Tres Provincias	C.M. Romero Rodriguez	769084 (MA)	JN663434	JN663655	JN663543	JQ814500	JQ814573
46	*L. filicaulis* Boiss. ex Leresche & Levier (2)	Spain: León. Canseco	C.M. Romero Rodriguez	789283 (MA)	JN663435	JN663656	JN663544	JX481118	-
47	*L. filicaulis* Boiss. ex Leresche & Levier (3)	Spain: León. Puerto del Pontón	F. Llamas & al.	619920 (MA)	JN663436	JN663657	JN663545	KF623191	-

48	*L. glacialis* Boiss. (1)	Spain: Granada. Sierra Nevada. Corral del Veleta	J.L. Blanco Pastor	43JB09	JN663437	JN663659	JN663547	JQ814504	JQ814577
49	*L. glacialis* Boiss. (2)	Spain: Granada. Sierra Nevada. El Caballo	J.L. Blanco Pastor	70JB09	JN663438	JN663660	JN663548	JQ814505	JQ814578
50	*L. glauca* (L.) Chaz. ssp. *glauca* (1)	Spain: Madrid. Colmenar de Oreja	J. Calvo	790863 (MA)	JN663439	JN663661	JN663549	JX481116	-
51	*L. glauca* (L.) Chaz. ssp. *glauca* (2)	Spain: Toledo. Ontígola	A. Segura	2009.12.148 (RNG)	JN663440	JN663662	JN663550	KF623192	-
52	*L. glauca* ssp. *olcadium* Valdés & D.A. Webb	Spain: Albacete. Balazote	Rivas Goday	2009.12.151 (RNG)	JN663441	JN663663	JN663551	JQ814506	JQ814579
53	*L. huteri* Lange.	Spain: Málaga. Sierra de Mijas, Mijas	J.L. Blanco Pastor	32JB09	JN663442	JN663664	JN663552	JX481111	-
54	*L. intricata* Coincy	Porgutal: Freixo-de-espada. Cinta	A. Teixeira	s.n.	JN663427	JN663648	JN663536	-	-
55	*L. lilacina* Lange. (1)	Spain: Granada. Baza	P. F. Cannon et al.	2009.12.38 (RNG)	JN663443	JN663666	JN663554	KF623194	-
56	*L. lilacina* Lange. (2)	Spain: Jaén. Otiñar	J.L. Blanco Pastor	2E1JB12	-	-	-	JX481156	KC625812
57	*L. micrantha* (Cav.) Hoffmanns. & Link. (1)	Spain: Badajoz. Almendralejo	J.L. Pérez	129272 (SEV)	JN663445	JN663668	JN663556	KF623195	-
58	*L. micrantha* (Cav.) Hoffmanns. & Link. (2)	Spain: Huelva. Marismas del Odiel	J.L. Blanco Pastor	22JB09	JN663446	JN663669	JN663557	JQ814513	JQ814585
59	*L. munbyana* Boiss. & Reut.	Spain: Huelva. Marismas del Odiel	J.L. Blanco Pastor	21JB09	JN663447	JN663670	JN663558	JQ814515	JQ814587
60	*L. oblongifolia* (Boiss.) Boiss. & Reut. ssp. *oblongifolia*	Spain: Málaga. El Torcal de Antequera	J.L. Blanco Pastor	34JB09	JN663449	JN663672	JN663560	JQ814516	JQ814588
61	*L. oblongifolia* ssp. *benitoi* (Fern. Casas) L. Sáez, M.B. Crespo, Juan & M.Bernal	Spain: Almería. Níjar	J. Calvo & al.	805599 (MA)	JN663491	JN663714	JN663602	KF623196	-
62	*L. oblongifolia* ssp. *haenseleri* (Boiss. & Reut.) Valdés.	Spain: Huelva. Peñas de Aroche	E. Sánchez-Gullón	s.n.	JN663448	JN663671	JN663559	KF623197	-
63	*L. oligantha* ssp. *oligantha* Lange. (1)	Spain: Alicante. Muro d'Alcoi	L. Serra	753096 (MA)	JN663450	JN663673	JN663561	JX481121	-
64	*L. oligantha* ssp. *oligantha* Lange. (2)	Spain: Almeria. Venta de los Yesos	E.F. Galiano et al.	11483 (SEV)	JN663451	JN663674	JN663562	KF623198	-
65	*L. orbensis* Carretero & Boira. (1)	Spain: Alicante. Orba	J.L. Blanco Pastor	3JB10	JN663452	JN663675	JN663563	KF623199	-
66	*L. orbensis* Carretero & Boira. (2)	Spain: Alicante. Sagra	J.L. Blanco Pastor	4JB10	JN663453	JN663676	JN663564	JQ814518	JQ814590
67	*L. platycalyx* Boiss.	Spain: Cádiz. Zahara de la Sierra	S. Martín Bravo	5SMB08	JN663454	JN663677	JN663565	JQ814520	JQ814592
68	*L. polygalifolia* Hoffmanns. & Link. ssp. *polygalifolia*	Portugal: Estremadura. Guincho	H.J.M. Bowen	2009.12.108 (RNG)	JN663457	JN663680	JN663568	JQ814523	JQ814479 JQ814483
69	*L. polygalifolia* ssp. *aguillonensis* (García Mart.) Castrov. & Lago	Spain: A Coruña. Punta Candelaria	J. Guitián	122145 (SEV)	JN663455	JN663678	JN663566	KF623200	-
70	*L. polygalifolia* ssp. *lamarckii* (Rouy) D.A. Sutton.	Portugal: Algarve. Monte Gordo	J.L. Blanco Pastor	33JB10	JN663456	JN663679	JN663567	JQ814522	JQ814594
71	*L. propinqua* Boiss. & Reut. (1)	Spain: Álava. Asparrena, Lizarrate	P.M. Uribe-Echebarría	753221 (MA)	JN663458	JN663681	JN663569	KF623201	-
72	*L. propinqua* Boiss. & Reut. (2)	Spain: Vizcaya. Zeanuri	J.A. Alejandre	468162 (MA)	JN663459	JN663682	JN663570	JQ814524	JQ814595

73	*L. saturejoides* Boiss. ssp. *saturejoides* (1)	Spain: Málaga. Sierra de Mijas. Mijas	J.L. Blanco Pastor	31JB09	JN663461	JN663684	JN663572	KF623202	-
74	*L. saturejoides* Boiss. ssp. *saturejoides* (2)	Spain: Málaga. Sierra Tejeda. Canilllas de Aceituno	J.L. Blanco Pastor	36JB09	JN663462	JN663685	JN663573	JQ814525	JQ814596
75	*L. saxatilis* (L.) Chaz. (1)	Spain: Madrid. Miraflores de la Sierra	P. Vargas	20PV09	JN663463	JN663686	JN663574	JX481115	-
76	*L. saxatilis* (L.) Chaz. (2)	Spain: Ávila. Hoyos del Espino	P. Vargas	94PV09	JN663464	JN663687	JN663575	JQ814526	JQ814597
77	*L. simplex* Willd. ex Desf. (1)	Turkey: Gümüshane. Tirebolu	C. Aedo & al.	688145 (MA)	JN663465	JN663688	JN663576	-	-
78	*L. simplex* Willd. ex Desf. (10)	Bulgaria: Nova Lovcha	A. Quintanar & al.	726863 (MA)	JN663466	JN663689	JN663577	-	-
79	*L. simplex* Willd. ex Desf. (11)	Italy: Abruzzo	C. Aedo & al.	698000 (MA)	JN663467	JN663690	JN663578	-	-
80	*L. simplex* Willd. ex Desf. (12)	Tunisia: Kasserine. Sbeïtla	J. Calvo & al.	798384 (MA)	JN663468	JN663691	JN663579	-	-
81	*L. simplex* Willd. ex Desf. (13)	France: Tournes	J. Lambinon	466422 (MA)	JN663469	JN663692	JN663580	-	-
82	*L. simplex* Willd. ex Desf. (2)	Morocco: Agadir-Melloul	Unknown collector	s.n.	JN663470	JN663693	JN663581	-	-
83	*L. simplex* Willd. ex Desf. (3)	Armenia. Syunik. Goris	G. Fayvush	790151 (MA)	JN663471	JN663694	JN663582	-	-
84	*L. simplex* Willd. ex Desf. (4)	Cyprus: Nicosia. Nisou	J. Lambinon	554534 (MA)	JN663472	JN663695	JN663583	-	-
85	*L. simplex* Willd. ex Desf. (5)	Morocco: Adrar-n-Oukaïmeden	A. Hierro & al.	746659 (MA)	JN663473	JN663696	JN663584	-	-
86	*L. simplex* Willd. ex Desf. (6)	Greece: Ararchova	P. Vargas	79PV08	JN663474	JN663697	JN663585	JQ814528	JQ814599
87	*L. simplex* Willd. ex Desf. (7)	Spain: Ávila. Padiernos	M. Fernández-Mazuecos	24MF08	JN663475	JN663698	JN663586	-	-
88	*L. simplex* Willd. ex Desf. (8)	Spain: Granada. Orjiva	R.N. Carter	2009.12.162 (RNG)	JN663476	JN663699	JN663587	JQ814527	JQ814598
89	*L. simplex* Willd. ex Desf. (9)	Spain: Alicante. Petrel	J. L. Blanco Pastor	5JB10	JN663477	JN663700	JN663588	-	-
90	*L. supina* (L.) Chaz. ssp. *supina* (1)	Spain: León. Puerto de San Glorio	M. Fernández-Mazuecos	38MF08	JN663479	JN663702	JN663590	-	-
91	*L. supina* (L.) Chaz. ssp. *supina* (2)	France: Gorges de l'Hérault	J. Lambinon	2009.12.131 (RNG)	JN663481	JN663704	JN663592	JQ814530	JQ814601
92	*L. supina* ssp. *maritima* (Lam. & DC.) M. Laínz	Spain: Vizcaya. La Arena	J. Loidi	2009.12.113 (RNG)	JN663480	JN663703	JN663591	KF623203	-
93	*L. thymifolia* (Vahl) DC.	France: Gironde. Carcans	B. de Retz	303566 (MA)	JN663482	JN663705	JN663593	KF623204	-
94	*L. tristis* (L.) Mill. ssp. *tristis*	Spain: Cádiz. Alcalá de los Gazules	P. Jiménez Mejías	105PJM04	JN663490	JN663713	JN663601	JX481109	KC625808
95	*L. tristis* ssp. *lurida* (Pau& Font Quer) Maire.	Morocco. Ksar-es-Souk. Akeimeden, Midkane	A. Charpin & AL.	330588 (MA)	JN663484	JN663707	JN663595	-	-
96	*L. tristis* ssp. *mesatlántica* D.A. Sutton. (1)	Morocco: Ifrane. Ifrane	M. Fernández-Mazuecos	11MF08	JN663485	JN663708	JN663596	KF623205	-
97	*L. tristis* ssp. *mesatlántica* D.A. Sutton. (2)	Morocco: Beni Mellal. El Ksiba	A. Quintanar & al.	745813 (MA)	JN663486	JN663709	JN663597	-	-
98	*L. tristis* ssp. *mesatlántica* D.A. Sutton. (3)	Morocco: Bou Teguerouine	A. Hierro & al.	746944 (MA)	JN663489	JN663712	JN663600	-	-
99	*L. tristis* ssp. *pectinata* (Pau & Font Quer)	Morocco: Tánger. Bab Taza	S. Andrés & al.	782480 (MA)	JN663487	JN663710	JN663598	KF623206	-

	Maire. (1)								
100	*L. tristis* ssp. *pectinata* (Pau & Font Quer) Maire. (2)	Morocco: Chefchaouene. Cherafat	J. Fernández Casas	330561 (MA)	JN663488	JN663711	JN663599	-	-
101	*L. tursica* Valdés & Cabezudo.	Spain: Huelva. Coto de Doñana	J.L. Blanco Pastor	18JB09	JN663492	JN663715	JN663603	JQ814533	JQ814603
102	*L. verticillata* Boiss. (1)	Spain: Granada. Sierra Nevada. Las Sabinas	J.M. Losa	2009.12.53 (RNG)	JN663493	JN663716	JN663604	JX481110	KC625810 KC625811
103	*L. verticillata* Boiss. (2)	Spain: Granada. Sierra Nevada. Pradollano	P. Vargas	75PV09	JN663494	JN663717	JN663605	KF623207	-
	Outgroup:								
104	*Antirrhinum graniticum* Rothm.	Spain: Cáceres. Trujillo	P. Vargas	213PV06	JF694120	JN663609	JN663497	JQ814484	JQ814556
105	*Chaenorhinum.macropodum* (Boiss. & Reut.) Lange	Spain: Málaga. Cómpeta	M. Fernández-Mazuecos	7E3MF08	JF694119	JN663610	JN663498	JQ814485	JQ814557
106	*L. chalepensis* (L.) Mill. (Sect. Macrocentrum)	Cyprus: Larnaca, Cape Kiti	Iter Mediterraneum IV	495681(MA)	JF694128	JN663647	JN663535	JQ814497	JQ814570
107	*L. triornithophora* (L.) Willd. (Sect. Pelisserianae)	Spain: León. Palacios del Sil	Martín-Blanco	617622(MA)	JN663483	JN663706	JN663594	-	-
108	*L. triornithophora* (L.) Willd. (Sect. Pelisserianae) (2)	Spain: Cáceres. Sierra de Gata	M. Fernández-Mazuecos	18MF07	-	-	-	JX481083	-
109	*L. elegans* Cav. (Sect. Versicolores)	Spain: Cuenca. El Tobar	M. Fernández-Mazuecos	55MF09	JN663433	JN663654	JN663542	KF623190	-
110	*L. gharbensis* Batt. & Pit. (Sect. Versicolores)	Spain: Huelva. Gibraleón	M. Fernández-Mazuecos	7MF09	JF694139	JN663658	JN663546	JQ814503	JQ814576
111	*L. multicaulis* (L.) Mill. (Sect. Versicolores)	Morocco: Azrou	M. Fernández-Mazuecos	15MF08	JF694155	JN663719	JN663607	JQ814514	JQ814586
112	*L. incarnata* (Vent.) Sprengel (Sect. Versicolores)	Spain: Badajoz. Alburquerque	M.Fernández-Mazuecos	9MF09	JF694145	JN663665	JN663553	KF623193	-
113	*L. spartea* (L.) Chaz. (Sect. Versicolores)	Spain: Cáceres. Monfragüe	M. Fernández-Mazuecos	4MF08	JN663478	JN663701	JN663589	JQ814529	JQ814600
114	*L. vulgaris* Mill. (Sect. Linaria)	France: Chamonix	B. Estébanez	s.n.	JQ814621	JQ814554	KF623221	JQ814535	JQ814477 JQ814481
115	*L. nivea* Boiss. & Reuter (Sect. Speciosae)	Spain: Toledo	C. Aedo	611701 (MA)	KF623211	KF623216	KF623220	JX481155	-
116	*L. repens* (L.) Mill. (Sect. Speciosae)	Spain: Cuenca. El Tobar	M. Fernández-Mazuecos	54MF09	KF623210	KF623215	KF623219	JX481144	-
117	*L. purpurea* (L.) Mill. (Sect. Speciosae)	United Kingdom: Norwich (cultivated)	M. Fernández-Mazuecos	74MF09	KF623208	KF623213	KF623217	JX481147	-
118	*L. reflexa* (L.) Chaz. (Sect. Diffusae)	Tunisia: Jerid, Cedada	C. Aedo et al.	795183 (MA)	KF623209	KF623214	KF623218	-	-
119	*L. reflexa* (L.) Chaz. (Sect. Diffusae) (2)	Algeria: Algiers	J.J. Aldasoro	A9799	-	-	-	JX481126	-
120	*L. texana* Scheele	EE.UU.: California. San Diego	D.E. Breedlove	494665 (MA)	JN663496	JN663720	JN663608	-	-

Table S2

	Plastid			Nuclear	
	***rpl32-trnL*^{UAG}**	***trnS*^{GCU}*-trnG*^{UUC}**	***trnL*^{UAA}*-trnF*^{GAA}**	**ITS (ITS1-5.8S-ITS2)**	**AGT1 intron**
Description	*intergenic spacer of SSC chloroplast region*	*intergenic spacer of LSC chloroplast region*	*intergenic spacer of LSC chloroplast region*	*Internal transcribed spacer 1 and 2 and 5.8S ribosomal RNA*	*Partial intron of the alanine glyoxylate aminotransferase (photorespiratory enzyme)*
References	Shaw *et al.* (2007)	Hamilton (1999)	Taberlet *et al.* (1991)	White (1990)	Liepman & Olsen (2001)
No. of previously published sequences used (unphased)	38. Blanco-Pastor *et al.* (2012)	38. Blanco-Pastor *et al.* (2012)	0	38. Blanco-Pastor *et al.* (2012) 18. Fernández-Mazuecos *et al.* (2013)	38. Blanco-Pastor *et al.* (2012) 4. Blanco-Pastor *et al.* (2013)
No. of newly generated sequences used (unphased)	79	79	117	36	1
Total number of sequences in the alignment (phased)	117	117	117	184	86
Aligned length (bp)	1742 (combined)			599	658
Ungapped length range	1337-1535 (combined)			571-592	379-542
% Identical sites	442 (25.4%) (combined)			299 (49.9%)	46(7.0%)
% Pairwise identity	91.6% (combined)			93.2%	82.1%
Variable characters	468 (combined)			233	320
Parsimony-informative characters	225 (combined)			215	311
Mean % G+C content	29.9% (combined)			57.9%	22.9%
Substitution model	GTR+I+G (combined)			HKY+G	HKY+G

Table S3

Taxon	Location	Haplotype numbers of *ssArv*	Haplotype numbers of *ssSax*	Haplotype numbers of *ssSup*
L. aeruginea (Gouan) Cav. ssp. *aeruginea* (1)	Spain: Granada. Sierra Nevada. Pradollano	-	-	**21**
L. aeruginea (Gouan) Cav. ssp. *aeruginea* (2)	Spain: Jaén. Sierra de Cazorla	-	-	**22**
L. aeruginea (Gouan) Cav. ssp. *aeruginea* (3)	Spain: Albacete. Yeste	-	-	**22**
L. aeruginea ssp. *nevadensis* (Boiss.) D.A. Sutton (1)	Spain: Almería. Sierra Nevada. Abrucena	-	-	**23**
L. aeruginea ssp. *nevadensis* (Boiss.) D.A. Sutton (2)	Spain: Granada. Sierra Nevada. Pico del Veleta	-	-	**22**
L. almijarensis Campo & Amo. (1)	Spain: Cordoba. Sierra Gallinera	-	-	**20**
L. almijarensis Campo & Amo. (2)	Spain: Córdoba. Priego de Cordoba. Las Angosturas	-	-	**31**
L. almijarensis Campo & Amo. (3)	Spain: Córdoba. Cabra. El Picacho	-	-	**22**
L. almijarensis Campo & Amo. (4)	Spain: Sevilla. Algámitas	-	-	**26**
L. alpina (L.) Mill. (1)	Slovenia: Karavanke. Zelenica-Palec	-	**4**	-
L. alpina (L.) Mill. (2)	Spain: Huesca. Formigal	-	**5**	-
L. amethystea Hoffmanns. & Link (Vent.) ssp. *amethystea* (1)	Spain: Málaga. Coín	-	**16**	-
L. amethystea Hoffmanns. & Link (Vent.) ssp. *amethystea* (2)	Spain: Ciudad Real. Ciudad Real	-	**21**	-
L. amethystea ssp. *broussonetii* (Poiret) Malato-Beliz	Morocco: Ifrane. Ifrane	-	**13**	-
L. amoi Campo ex Amo (1)	Spain: Sierra Tejeda. Canillas de Aceituno	-	-	**22**
L. amoi Campo ex Amo (2)	Spain: Málaga. Cómpeta	-	-	**22**
L. anticaria Boiss. & Reut.	Spain: Málaga. El Torcal de Antequera	-	-	**22**
L. arenaria DC.	France. Vendée. SintGilles	-	**15**	-
L. arvensis (L.) Desf. (1)	France: Córse. Col de Bigorno.	**2**	-	-
L. arvensis (L.) Desf. (2)	Spain: Almería. Sierra de los Filabres	**1**	-	-
L. arvensis (L.) Desf. (3)	Morocco: Souss-Massa. Daraâ	**1**	-	-
L. arvensis (L.) Desf. (4)	Spain: Soria. El Royo	**3**	-	-
L. atlantica Boiss. & Reuter	Morocco: Kebdana	-	-	-
L. atrofusca (Rouy) (1)	Spain: Ávila. Mengamuñoz.	-	-	**10**
L. atrofusca (Rouy) (2)	Spain: Cuenca. El Tobar	-	-	**10**

L. atrofusca (Rouy) (3)	Spain: Guadalajara. Póveda	-	-	**11**
L. atrofusca (Rouy) (4)	Spain: Madrid. San Lorenzo del Escorial	-	-	**10**
L. atrofusca (Rouy) (5)	Spain: Ciudad Real. Villanueva de la Fuente	-	-	**12**
L. badalii Loscos *(*1)	Spain: Huesca. Sopeira	-	**7**	-
L. badalii Loscos (2)	Spain: León. Riaño	-	**11**	-
L. badalii Loscos (3)	Spain: Burgos. Pancorbo	-	**16**	-
L. bipunctata (L.) Chaz. ssp. *bipunctata* (1)	Spain: Madrid. Sierra de Guadarrama	-	**19**	-
L. bipunctata (L.) Chaz. ssp. *bipunctata* (2)	Spain: Soria. Quintana Redonda	-	**18**	-
L. bubani Font Quer	Spain: Huesca. El Pueyo de Araguás	-	**12**	-
L. caesia (Pers.) F.G.Dietr. (1)	Spain: Ciudad Real. Daimiel	-	-	**8**
L. caesia (Pers.) F.G.Dietr. (2)	Spain: Soria. Almazán	-	-	**7**
L. cuartanensis (Degen & Hervier) Fern. Casas ex. Blanco-Pastor (1)	Spain: Granada. Sierra de Guillimona	-	-	**7**
L. cuartanensis (Degen & Hervier) Fern. Casas ex. Blanco-Pastor (2)	Spain: Albacete. Yeste	-	-	**25**
L. cuartanensis (Degen & Hervier) Fern. Casas ex. Blanco-Pastor (3)	Spain: Granada. Sierra de la Sagra	-	-	**22**
L. depauperata Leresche ex Lange ssp. *depauperata*	Spain: Alicante. Alcoi	-	-	**22**
L. depauperata ssp. *hegelmaieri* (Lange) De la Torre, Alcaraz & M.B. Crespo (1)	Spain: Alicante. Petrel	-	-	**29**
L. depauperata ssp. *hegelmaieri* (Lange) De la Torre, Alcaraz & M.B. Crespo (2)	Spain: Albacete. Fábrica de Riópar.	-	-	**25**
L. depauperata ssp. *ilergabona* (M.B. Crespo & Arán) L. Sáez	Spain: Castellón. Vistabella	-	-	**10**
L. diffusa Hoffmanns. & Link.	Portugal: Freixo-de-espada à Cinta	-	**16**	-
L. filicaulis Boiss. ex Leresche & Levier (1)	Spain: León. Pico Tres Provincias	-	**3**	-
L. filicaulis Boiss. ex Leresche & Levier (2)	Spain: León. Canseco	-	**3**	-
L. filicaulis Boiss. ex Leresche & Levier (3)	Spain: León. Puerto del Pontón	-	**3**	-
L. glacialis Boiss. (1)	Spain: Granada. Sierra Nevada. Corral del Veleta	-	-	**14**
L. glacialis Boiss. (2)	Spain: Granada. Sierra Nevada. El Caballo	-	-	**17**
L. glauca (L.) Chaz. ssp. *glauca* (1)	Spain: Madrid. Colmenar de Oreja	-	**8**	-
L. glauca (L.) Chaz. ssp. *glauca* (2)	Spain: Toledo. Ontígola	-	**9**	-
L. glauca ssp. *olcadium* Valdés & D.A. Webb	Spain: Albacete. Bazalote	-	**20**	-
L. huteri Lange.	Spain: Málaga. Sierra de Mijas	-	**16**	-

L. intricata Coincy	Porgutal: Freixo-de-espada. Cintra	-	**22**	-
L. lilacina Lange.	Spain: Granada. Baza	-	-	**22**
L. micrantha (Cav.) Hoffmanns. & Link. (1)	Spain: Badajoz. Almendralejo	**10**	-	-
L. micrantha (Cav.) Hoffmanns. & Link. (2)	Spain: Huelva. Marismas del Odiel	**10**	-	-
L. munbyana Boiss. & Reut.	Spain: Huelva. Marismas del Odiel	-	**14**	-
L. oblongifolia (Boiss.) Boiss. & Reut. ssp. *oblongifolia*	Spain: Málaga. El Torcal de Antequera	-	-	**29**
L. oblongifolia ssp. *benitoi* (Fern. Casas) L. Sáez, M.B. Crespo, Juan & M.Bernal	Spain: Almería. Níjar	-	-	**30**
L. oblongifolia ssp. *haenseleri* (Boiss. & Reut.) Valdés.	Spain: Huelva. Peñas de Aroche	-	-	**29**
L. oligantha ssp. *oligantha* Lange. (1)	Spain: Alicante. Muro d'Alcoi	-	**1**	-
L. oligantha ssp. *oligantha* Lange. (2)	Spain: Almeria. Venta de los Yesos	-	**2**	-
L. orbensis Carretero & Boira. (1)	Spain: Alicante. Orba	-	**6**	-
L. orbensis Carretero & Boira. (2)	Spain: Alicante. Sagra	-	**6**	-
L. platycalyx Boiss.	Spain: Cádiz. Zahara de la Sierra	-	-	**22**
L. polygalifolia Hoffmanns. & Link. ssp. *polygalifolia*	Portugal: Estremadura. Guincho	-	-	**16**
L. polygalifolia s. aguillonensis (García Mart.) Castrov. & Lago	Spain: A Coruña. Punta Candelaria	-	-	**6**
L. polygalifolia ssp. *lamarckii* (Rouy) D.A. Sutton.	Portugal: Algarve. Monte Gordo	-	-	**28**
L. propinqua Boiss. & Reut. (1)	Spain: Álava. Asparrena	-	**10**	-
L. propinqua Boiss. & Reut. (2)	Spain: Vizcaya. Zeanuri	-	**10**	-
L. saturejoides Boiss. ssp. *saturejoides* (1)	Spain: Málaga. Sierra de Mijas. Mijas	-	-	**24**
L. saturejoides Boiss. ssp. *saturejoides* (2)	Spain: Málaga. Sierra Tejeda. Canillas de Aceituno	-	-	**29**
L. saxatilis (L.) Chaz. (1)	Spain: Madrid. Miraflores de la Sierra	-	**23**	-
L. saxatilis (L.) Chaz. (2)	Spain: Ávila. Hoyos del Espino	-	**17**	-
L. simplex Willd. ex Desf. (1)	Turkey: Gümüshane. Tirebolu	**4**	-	-
L. simplex Willd. ex Desf. (10)	Bulgaria: Nova Lovcha	**13**	-	-
L. simplex Willd. ex Desf. (11)	Italy: Abruzzo	**12**	-	-
L. simplex Willd. ex Desf. (12)	Tunisia: Kasserine. Sbeïtla	**11**	-	-
L. simplex Willd. ex Desf. (13)	France: Tournes	**11**	-	-
L. simplex Willd. ex Desf. (2)	Morocco: Agadir-Melloul	**4**	-	-

L. simplex Willd. ex Desf. (3)	Armenia. Syunik. Goris	**7**	-	-
L. simplex Willd. ex Desf. (4)	Cyprus: Nicosia. Nisou	**4**	-	-
L. simplex Willd. ex Desf. (5)	Morocco: Adrar-n-Oukaïmeden	**9**	-	-
L. simplex Willd. ex Desf. (6)	Greece: Ararchova	**8**	-	-
L. simplex Willd. ex Desf. (7)	Spain: Ávila. Padiernos	**14**	-	-
L. simplex Willd. ex Desf. (8)	Spain: Granada. Orjiva	**5**	-	-
L. simplex Willd. ex Desf. (9)	Spain: Alicante. Petrel	**6**	-	-
L. supina (L.) Chaz. ssp. *supina* (1)	Spain: León. Puerto de San Glorio	-	-	**5**
L. supina (L.) Chaz. ssp. *supina* (2)	France: Gorges de l'Hérault	-	-	**13**
L. supina ssp. *maritima* (Lam. & DC.) M. Laínz	Spain: Vizcaya. La Arena	-	-	**5**
L. thymifolia (Vahl) DC.	France: Gironde. Carcans	-	-	**19**
L. tristis (L.) Mill. ssp. *tristis*	Spain: Cádiz. Alcalá de los Gazules	-	-	**9**
L. tristis ssp. *lurida* (Pau& Font Quer) Maire.	Morocco. Ksar-es-Souk. Akeimeden, Midkane	-	-	**15**
L. tristis ssp. *mesatlántica* D.A. Sutton. (1)	Morocco: Ifrane. Ifrane	-	-	**2**
L. tristis ssp. *mesatlántica* D.A. Sutton. (2)	Morocco: Beni Mellal. El Ksiba	-	-	**1**
L. tristis ssp. *mesatlántica* D.A. Sutton. (3)	Morocco: Bou Teguerouine	-	-	**18**
L. tristis ssp. *pectinata* (Pau & Font Quer) Maire. (1)	Morocco: Tánger. Bab Taza	-	-	**4**
L. tristis ssp. *pectinata* (Pau & Font Quer) Maire. (2)	Morocco: Chefchaouene. Cherafat	-	-	**3**
L. tursica Valdés & Cabezudo.	Spain: Huelva. Doñana	-	-	-
L. verticillata Boiss. (1)	Spain: Granada. Sierra Nevada. Las Sabinas	-	-	**22**
L. verticillata Boiss. (2)	Spain: Granada. Sierra Nevada. Pradollano	-	-	**27**

Table S4

Taxa	Locality	num. of indiv.	num. of pollinated flowers (fruit set)			fruit/flower ratio			Breeding system significance based in flower manipulations	P/O ± SD.	Breeding system significance following Cruden (1977)	Data source
			SA	FA	FX	SA	FA	FX				
L. aeruginea (a)	Spain: Granada. Sierra Nevada	-	-	-	-	-	-	-	-	1379.28 ± 110.12	Facultative xenogamy	Present study
L. aeruginea (b)	Spain: Madrid. El Escorial	1	8(0)	-	-	0	-	-	Xenogamy	-	-	Valdés (1969)
L. almijarensis	Spain: Córdoba. Priego de Córdoba	1	>10(0)	-	-	0	-	-	Xenogamy	-	-	Valdés (1970b)
L. alpina (a)	-	1	10(0)	-	-	0	-	-	Xenogamy	-	-	Valdés (1970b)
L. alpina (b)	-	>4	-	-	-	-	-	-	Autogamy	-	-	Docherty (1982)
L. alpina (c)	-	-	-	-	-	-	-	-	Facultative autogamy	-	-	Bruun (1937)
L. amethystea (a)	Spain. Ávila. Hoyo de Pinares	2	>20(0)	-	10(10)	0	-	1	Xenogamy	-	-	Present study
L. amethystea (b)	-	-	-	-	-	-	-	-	Facultative xenogamy	-	-	Valdés (1970b)
L. amoi (a)	Spain: Málaga. Sierra Tejeda	6	13(0)	12(0)	4(3)	0	0	0.75	Xenogamy	2457.64 ± 162.30	Xenogamy	Present study
L. amoi (b)	Spain: Málaga. Sierra de Almijara	1	65(0)	-	-	0	-	-	Xenogamy	-	-	Valdés (1970b)
L. anticaria	Spain: Málaga. El Torcal de Antequera	17	25(0)	42(9)	24(19)	0	0.214	0.791	Facultative xenogamy	1531.76 ± 92.33	Facultative xenogamy	Present study
L. arenaria	-	-	-	-	-	-	-	-	Autogamy	-	-	Serrano Pérez & Carbajal Villaverde (2010)
L. arvensis	Spain. Ávila. Hoyo de Pinares	40	101(97)	-	-	0.960	-	-	Autogamy	44.76±11.05	Obligate autogamy	Present study (see also Champagnat 1961; Knuth 1909)
L. atlántica	-	-	-	-	-	-	-	-	-	-	-	-
L. atrofusca	Spain: Ávila. Cadalso de los Vidrios	4	46(1)	18(2)	7(6)	0.022	0.111	0.857	Facultative xenogamy	1412.30±123.32	Facultative xenogamy	Present study
L. badalii	-	-	-	-	-	-	-	-	-	-	-	-
L. bipunctata	-	-	-	-	-	-	-	-	-	-	-	-
L. bubanii	-	-	-	-	-	-	-	-	-	-	-	-
L. caesia	Spain: Madrid. Cerro Negro	1	30(0)	-	10(3)	0	-	0.333	Xenogamy	-	-	Valdés (1970b)
L. cuartanensis	-	-	-	-	-	-	-	-	-	-	-	-

L. depauperata	Spain: Alicante. Petrel.	15	53(4)	34(8)	27(17)	0.075	0.235	0.630	Facultative xenogamy	1441.75 ± 217.15	Facultative xenogamy	Present study
L. diffusa	-	-	-	-	-	-	-	-	-	-	-	-
L. filicaulis	Spain: Oviedo. Picos de Europa	2	>20(0)	-	10(10)	0	-	1	Xenogamy	-	-	Present study
L. glacialis	Spain: Granada. Sierra Nevada.	11	24(23)	2(2)	9(9)	0.958	1	1	Autogamy	1684.17 ± 268.17	Facultative xenogamy	Present study
L. glauca	-	-	-	-	-	-	-	-	-	-	-	-
L. huteri	Spain: Málaga. Sierra de Mijas	5	5(0)	-	-	-	-	-	Xenogamy	-	-	Present study
L. intricata	-	-	-	-	-	-	-	-	-	-	-	-
L. lilacina	Spain: Jaén. Sierra de Cazorla	27	121(6)	122(8)	121(109)	0.050	0.066	0.900	Facultative xenogamy	-	-	Sánchez-Lafuente, unpublished
L. micrantha	Spain: Madrid. Tres Cantos	16	54(39)	-	-	0.722	-	-	Autogamy	18.09±3.71	Obligate autogamy	Present study
L. munbyana	-	-	-	-	-	-	-	-	-	-	-	-
L. oblongifolia	Spain: Huelva. Niebla	-	-	-	-	-	-	-	-	1723.05 ± 353.81	Facultative xenogamy	Present study
L. oligantha	-	-	-	-	-	-	-	-	Xenogamy/Facultative xenogamy	-	-	Melendo *et al.*(2003)
L. orbensis	Spain: Alicante. Parcent	-	63(4)	-	33(31)*	0.0635	-	0.94	Facultative xenogamy	-	-	Herreros *et al.*(2004)
L. platycalyx	Spain: Cádiz. Zahara de la Sierra	10	24(2)	20(1)	12(9)	0.083	0.048	0.750	Facultative xenogamy	3861,58 ± 633.43	Xenogamy	Present study
L. polygalifolia	Spain: Huelva. Isla Canela	11	>100(0)	-	131(124)	0	-	0.947	Xenogamy	2276.00 ± 454.49	Xenogamy	Junta de Andalucía, unpublished and Present study
L. propinqua	-	-	-	-	-	-	-	-	-	-	-	-
L. saturejoides (a)	Spain: Málaga. Sierra Tejeda	7	11(0)	2(0)	6(6)	0	0	1	Xenogamy	1156.33 ± 169.74	Facultative xenogamy	Present study
L. saturejoides (b)	Spain: Málaga. Sierra de Mijas	12	48(0)	3(0)	11(7)	0	0	0.636	Xenogamy	-	-	Present study
L. saxatilis	Spain: Madrid. Sierra de Guadarrama	2	>100(0)	-	10(10)	0	-	1	Xenogamy	-	-	Present study
L. simplex (a)	Spain: Madrid. Rivas-Vaciamadrid	29	61(48)	-	-	0.787	-	-	Autogamy	37.93±5.27	Obligate autogamy	Present study
L. simplex (b)	-	1	35(20)	-	-	0.571	-	-	Autogamy	-	-	Valdés (1970b)
L. supina	-	1	>10(0)	-	-	0	-	-	Xenogamy	-	-	Valdés (1970b)
L. thymifolia	-	-	-	-	-	-	-	-	-	-	-	-
L. tristis (a)	Spain. Cádiz. Alcalá	7	8(0)	17(1)	8(6)	0	0.059	0.75	Facultative xenogamy	2577.06 ±	Xenogamy	Present study

	de los Gazules									133.79		
L. tristis (b)	Spain: Málaga. Montes de Tolox	3	91(15)	-	11(5)	0.164	-	0.454	Facultative xenogamy	-	-	Valdés (1970b)
L. verticillata	Spain: Granada. Sierra Nevada.	6	9(0)	13(0)	11(9)	0	0	0.818	Xenogamy	2022.18 ± 385.80	Xenogamy	Present study
L. tursica	Spain: Huelva. Doñana	40	40(35)	-	-	0.875	-	-	Autogamy	46.53 ± 9.40	Obligate autogamy	Valdés & Lifante (1996)

Pollination treatments: SA (spontaneous autogamy), FA (forced autogamy) and FX (forced xenogamy); *open pollination in field

Table S5

Taxa	Locality	No. of pollen grains per anther						No. of pollen grains per flower			No. of ovules per flower			Pollen-Ovule ratios				
					x dilution factor			x 4 (num of anthers)										
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	mean P/O	SD.
L. aeruginea (a)	Spain: Granada. Sierra Nevada.	1221	1154	1780	24420	23080	35600	97680	92320	142400	72	72	95	1356.67	1282.22	1498.95	1379.28	110.12
L. atrofusca	Spain: Ávila. Cadalso de los Vidrios	1743	1212	1590	34860	24240	31800	139440	96960	127200	92	76	88	1515.65	1275.79	1445.45	1412.30	123.32
L. amoi (a)	Spain: Málaga. Sierra Tejeda	2034	1592	2390	40680	31840	47800	162720	127360	191200	63	56	76	2582.86	2274.29	2515.79	2457.64	162.30
L. anticaria (a)	Spain: Málaga. El Torcal de Antequera	1998	2408	2558	39960	48160	51160	159840	192640	204640	98	133	135	1631.02	1448.42	1515.85	1531.76	92.33
L. arvensis	Spain: Ávila. Hoyo de Pinares	706	408	904				2824	1632	3616	55	51	71	51.35	32.00	50.93	44.76	11.05
L. depauperata	Spain: Alicante. Petrel.	2300	1775	2315	46000	35500	46300	184000	142000	185200	113	118	124	1628.32	1203.39	1493.55	1441.75	217.15
L. glacialis	Spain: Granada. Sierra Nevada.	2032	1463	1366	40640	29260	27320	162560	117040	109280	82	80	68	1982.44	1463.00	1607.06	1684.17	268.17
L. micrantha	Spain: Madrid. Tres Cantos	220	260	164				880	1040	656	48	48	46	18.33	21.67	14.26	18.09	3.71
L. oblongifolia	Spain: Huelva. Niebla	1320	1285	837	26400	25700	16740	105600	102800	66960	63	49	48	1676.19	2097.96	1395.00	1723.05	353.81
L. platycalyx	Spain: Cádiz. Zahara de la Sierra	2961	2155	2684	59220	43100	53680	236880	172400	214720	57	55	50	4155.79	3134.55	4294.40	3861.58	633.43
L. polygalifolia	Spain: Huelva. Isla Canela	1802	1901	2004	36040	38020	40080	144160	152080	160320	81	64	60	1779.75	2376.25	2672.00	2276.00	454.49
L. saturejoides (a)	Spain: Málaga. Sierra Tejeda	492	472	398	9840	9440	7960	39360	37760	31840	36	28	31	1093.33	1348.57	1027.10	1156.33	169.74
L. saturejoides (b)	Spain: Málaga. Sierra de Mijas	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L. simplex (a)	Spain: Madrid. Rivas-Vaciamadrid	685	850	760				2740	3400	3040	64	88	94	42.81	38.64	32.34	37.93	5.27
L. tristis (a)	Spain. Cádiz. Alcalá de los Gazules	3338	2387	2885	66760	47740	57700	267040	190960	230800	104	78	85	2567.69	2448.21	2715.29	2577.06	133.79
L. verticillata	Spain: Granada. Sierra Nevada.	2164	2201	2520	43280	44020	50400	173120	176080	201600	92	102	82	1881.74	1726.27	2458.54	2022.18	385.80

Table S6

Characters / Species	Breeding System (autogamous/xenogamous)*	Lifespan (annual/perennial)	Seed type (small wingless/ large winged)	Substrate requirements (wide variety/specific)*	Range expansion ability (high/low)**
L. aeruginea	Xenogamous	Perennial	Large winged seeds	Basic	Low
L. almijarensis	Xenogamous	Perennial	Large winged seeds	Basic	Low
L. alpina	Autogamous	Missing data	Large winged seeds	Basic or acid	High
L. amethystea	Xenogamous	Annual	Large winged seeds	Basic or acid (including disturbed landscapes)	Low
L. amoi	Xenogamous	Perennial	Large winged seeds	Basic	Low
L. anticaria	Xenogamous	Perennial	Large winged seeds	Basic	Low
L. arenaria	Autogamous	Annual	Missing data (marginal ridge)	Sandy	Low
L. arvensis	Autogamous	Annual	Large winged seeds	Basic or acid (including disturbed landscapes)	High
L. atlantica	Missing data	Annual	Small wingless seeds	Sandy	Low
L. atrofusca	Xenogamous	Perennial	Large winged seeds	Basic	Low
L. badalii	Missing data	Annual	Large winged seeds	Basic or acid	Low
L. bipunctata	Missing data	Annual	Missing data (marginal ridge)	Basic or acid (including disturbed landscapes)	Low
L. bubanii	Missing data	Annual	Large winged seeds	Basic	Low
L. caesia	Xenogamous	Perennial	Large winged seeds	Basic or acid (including disturbed landscapes)	Low
L. cuartanensis	Xenogamous	Perennial	Large winged seeds	Basic	Low
L. depauperata	Xenogamous	Perennial	Large winged seeds	Basic	Low
L. diffusa	Missing data	Annual	Large winged seeds	Basic or acid (including disturbed landscapes)	Low
L. filicaulis	Xenogamous	Missing data	Large winged seeds	Basic	Low
L. glacialis	Autogamous	Missing data	Large winged seeds	Acid	Low
L. glauca	Missing data	Missing data	Large winged seeds	Basic	Low
L. intricata	Missing data	Annual	Missing data (marginal ridge)	Acid	Low
L. lilacina	Xenogamous	Perennial	Large winged seeds	Basic	Low
L. micrantha	Autogamous	Annual	Large winged seeds	Basic or acid (including disturbed landscapes)	High

L. munbyana	Missing data	Annual	Large winged seeds	Sandy	Low
L. oligantha	Xenogamous	Annual	Missing data (marginal ridge)	Basic or acid (including disturbed landscapes)	Low
L. platycalyx	Xenogamous	Missing data	Large winged seeds	Basic	Low
L. polygalifolia	Xenogamous	Perennial	Large winged seeds	Sandy	Low
L. propinqua	Missing data	Missing data	Large winged seeds	Basic	Low
L. saxatilis	Xenogamous	Perennial	Large winged seeds	Basic or acid	Low
L. simplex	Autogamous	Annual	Large winged seeds	Basic or acid (including disturbed landscapes)	High
L. supina	Xenogamous	Perennial	Large winged seeds	Basic or acid (including disturbed landscapes)	Low
L. thymifolia	Missing data	Perennial	Large winged seeds	Sandy	Low
L. tristis	Xenogamous	Perennial	Large winged seeds	Basic	Low
L. tursica	Autogamous	Annual	Small wingless seeds	Sandy	Low
L. verticillata	Xenogamous	Perennial	Large winged seeds	Basic	Low
Data source	Present study and others (see Table S4)	Sutton (1988) and Sáez & Bernal (2009)			Range sizes obtained from GBIF; http://www.gbif.org , ANTHOS; http://www.anthos.es and Valdés (1970a)

*Wide variety of substrates considered when indifferent to substrate (acid or basic) and including those of disturbed landscapes
** High range expansion ability considered when distributed in >2 areas as delimited in the biogeographic analysis

Table S7

			Breeding System		**Lifespan**		**Seed type**		**Substrate requirements**	
			Autogamous	Xenogamous	Annual	Perennial	Small wingless seeds	Large winged seeds	Wide variety of substrates	Specific substrates
Lifespan	Annual	*m* statistic	0.08 (≤0.001***)	-0.05 (≤0.001***)	-	-	-	-	-	-
		d statistic	0.07 (≤0.001***)	-0.07 (≤0.001***)	-	-	-	-	-	-
	Perennial	*m* statistic	-0.03 (0.01**)	0.09 (≤0.001***)	-	-	-	-	-	-
		d statistic	-0.07 (≤0.001***)	0.07 (≤0.001***)	-	-	-	-	-	-
Seed type	Small wingless seeds	*m* statistic	$6x10^{-3}$ (≤0.001***)	$-4x10^{-3}$ (≤0.001***)	0.01 (≤0.001***)	$-5x10^{-3}$ (0.02*)	-	-	-	-
		d statistic	$5x10^{-3}$ (≤0.001***)	$-5x10^{-3}$ (≤0.001***)	$9x10^{-3}$ (≤0.001***)	$-9x10^{-3}$ (≤0.001***)	-	-	-	-
	Large winged seeds	*m* statistic	$-5x10^{-3}$ (≤0.001***)	$5x10^{-3}$ (≤0.001***)	$-8x10^{-3}$ (≤0.001***)	0.01 (≤0.001***)	-	-	-	-
		d statistic	$-5x10^{-3}$ (≤0.001***)	$5x10^{-3}$ (≤0.001***)	$-9x10^{-3}$ (≤0.001***)	$9x10^{-3}$ (≤0.001***)	-	-	-	-
Substrate requirements	Wide substrate requirements	*m* statistic	$1x10^{-4}$ (NS)	$1x10^{-5}$ (NS)	$1x10^{-5}$ (≤0.001***)	$-1x10^{-5}$ (≤0.001***)	$4x10^{-5}$ (NS)	$-1x10^{-4}$ (NS)	-	-
		d statistic	$2x10^{-6}$ (NS)	$-6x10^{-5}$ (NS)	$1x10^{-5}$ (≤0.001***)	$-1x10^{-5}$ (≤0.001***)	$-7x10^{-5}$ (NS)	$-2x10^{-4}$ (NS)	-	-
	Specific substrate requirements	*m* statistic	$2x10^{-5}$ (NS)	$2x10^{-5}$ (NS)	$-1x10^{-5}$ (≤0.001***)	$1x10^{-5}$ (≤0.001***)	$-7x10^{-5}$ (NS)	$-2x10^{-4}$ (NS)	-	-
		d statistic	$-6x10^{-5}$ (NS)	$-3x10^{-5}$ (NS)	$-1x10^{-5}$ (≤0.001***)	$1x10^{-5}$ (≤0.001***)	$-1x10^{-4}$ (NS)	$-3x10^{-4}$ (NS)	-	-
Range expansion ability	High	*m* statistic	0.06 (0.04*)	-0.01 (NS)	0.03 (0.03*)	$5x10^{-5}$ (NS)	$2x10^{-4}$ (NS)	$7x10^{-4}$ (NS)	$7x10^{-4}$ (0.04*)	$-4x10^{-4}$ (0.05*)
		d statistic	0.03 (0.05*)	-0.03 (0.05*)	0.03 (0.04*)	-0.03 (0.04*)	$-1x10^{-4}$ (NS)	$1x10^{-4}$ (NS)	$5x10^{-4}$ (0.04*)	$-5x10^{-4}$ (0.05*)
	Low	*m* statistic	-0.03 (0.04*)	0.04 (0.05*)	-0.02 (0.02*)	0.03 (0.04*)	$3x10^{-4}$ (NS)	$-1x10^{-5}$ (NS)	$-5x10^{-4}$ (NS)	$5x10^{-4}$ (0.04*)
		d statistic	-0.03 (0.05*)	0.03 (0.05*)	-0.03 (0.04*)	0.03 (0.04*)	$1x10^{-4}$ (NS)	$-1x10^{-4}$ (NS)	$-5x10^{-4}$ (NS)	$5x10^{-4}$ (0.04*)

Significance codes: (NS) not significant; * ≤0.05; **≤0.01;*** ≤0.001

References

Blanco-Pastor JL, Fernández-Mazuecos M, Vargas P (2013) Past and future demographic dynamics of alpine species: limited genetic consequences despite dramatic range contraction in a plant from the Spanish Sierra Nevada. *Molecular Ecology* **22**, 4177-4195.

Blanco-Pastor JL, Vargas P, Pfeil BE (2012) Coalescent Simulations Reveal Hybridization and Incomplete Lineage Sorting in Mediterranean *Linaria*. *Plos One* **7**, e39089.

Bruun HG (1937) Genetical notes on *Linaria*, I-II. *Hereditas* **22**, 295-400.

Champagnat M (1961) Recherches de morphologie descriptive et expérimentale sur le genre *Linaria*. *Ann Sci Nat Bot (Paris)* **12**, 1-170.

Cruden RW (1977) Pollen-Ovule Ratios: A Conservative Indicator of Breeding Systems in Flowering Plants. *Evolution* **31**, 32-46.

Docherty Z (1982) Self-incompatibility in *Linaria*. *Heredity* **49**, 349-352.

Fernández-Mazuecos M, Blanco-Pastor JL, Vargas P (2013) A Phylogeny of Toadflaxes (*Linaria* Mill.) Based on Nuclear Internal Transcribed Spacer Sequences: Systematic and Evolutionary Consequences. *International Journal of Plant Sciences* **174**, 234-249.

Hamilton MB (1999) Four primer pairs for the amplification of chloroplast intergenic regions with intraspecific variation. *Molecular Ecology* **8**, 521-523.

Herreros R, Carrió E, Güemes J (2004) Study of the populations of *Linaria orbensis* Carretero & Boira: basis for a strategy of conservation. In: *4th European Conference on the Conservation of Wild Plants*, Valencia (Spain).

Knuth P (1909) *Handbook of Flower Pollination: Based Upon Hermann Müller's Work 'The Fertilisation of Flowers by Insects'* Clarendon Press.

Liepman AH, Olsen LJ (2001) Peroxisomal alanine : glyoxylate aminotransferase (AGT1) is a photorespiratory enzyme with multiple substrates in *Arabidopsis thaliana*. *The Plant Journal* **25**, 487-498.

Melendo M, Giménez E, Cano E, Mercado FG, Valle F (2003) The endemic flora in the south of the Iberian Peninsula: taxonomic composition, biological spectrum, pollination, reproductive mode and dispersal. *Flora - Morphology, Distribution, Functional Ecology of Plants* **198**, 260-276.

Sáez L, Bernal M (2009) *Linaria* Mill. In: *Flora Iberica 13* (eds. Castroviejo S, Herrero A, Benedí C, Rico E, Güemes J), pp. 232-324. CSIC, Madrid.

Serrano Pérez M, Carbajal Villaverde R (2010) *Linaria arenaria* DC. In: *Atlas y Libro Rojo de la Flora Vascular Amenazada de España* (eds. Bañares Á, Blanca G, Güemes J, Moreno JC, Ortiz S). Ministerio de Medio Ambiente y Medio Rural y Marino.

Shaw J, Lickey EB, Schilling EE, Small RL (2007) Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: The Tortoise and the hare III. *American Journal of Botany* **94**, 275-288.

Sutton DA (1988) *A revision of the tribe Antirrhineae* Oxford University Press, London. UK.

Taberlet P, Gielly L, Pautou G, Bouvet J (1991) Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology* **17**, 1105-1109.

Valdés B (1969) *Taxonomía experimental del género Linaria Miller y sistemática de las especies europeas de semilla alada. Tesis doctoral.*, Universidad Complutense de Madrid.

Valdés B (1970a) *Revisión de las especies europeas de Linaria con semillas aladas.* Anales de la Universidad Hispalense, Sevilla. Spain.

Valdés B (1970b) Taxonomía experimental del género *Linaria* IV. Reproducción sexual: autogamia y polinización intraespecífica. *Bol. R. Soc. Española Hist. Nat. (Biol.)* **68**, 79-89.

Valdés B, Lifante ZD (1996) Habitual autogamy in *Linaria tursica* Valdés et Cabezudo (Scrophulariaceae). *Flora* **191**, 329-333.

White TJ, Bruns, T., Lee, S. and Taylor, J. (1990) *Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In "PCR Protocols. A Guide to Methods and Applications"* Academic Press, San Diego. USA.

Supplementary Materials and Methods

Species tree inference

The *BEAST analysis requires allelic data, here we used the Bayesian statistical method of PHASE 2.1 (Stephens et al. 2001; Stephens & Donnelly 2003) as implemented in DnaSP v5 (Librado & Rozas 2009) for the haplotype deciphering of the newly generated (unphased) ITS sequences. The phasing analysis was carried out with default parameters.

The *BEAST analysis was carried out with similar prior settings and calibration dates as used in Blanco-Pastor et al. (2012). Additionally, here we included a prior for the population hyper-parameter operator (species.popMean) with normal distribution (mean = 0.09, Stdev = 0.03) as obtained from the posterior distribution of the *BEAST species tree analysis in Blanco-Pastor et al. (2012) (all putative hybrids excluded). Thirty Markov Chain Monte Carlo (MCMC) analyses were run for 100 million generation each, with a sample frequency of 40000. Convergence of parameters and adequate sample sizes was confirmed with Tracer 1.5 (Rambaut 2009), with ESS values above 200. Runs were combined using LogCombiner v.1.7.3, after discarding the first 10% of sampled generations as burn-in. Trees were summarized in a maximum clade credibility (MCC) species tree obtained in TreeAnotator v.1.7.3 and visualized in FigTree v.1.3.1. In order to visualize the temporal dynamics of diversification in *Linaria* sect. *Supinae,* we generated lineage-through-time (LTT) plots in the R package *ape* (Paradis *et al.* 2004). We used the MCC species tree and a random sample of 1000 trees from the posterior distribution of the *BEAST analysis. All trees were previously pruned to exclusively contain *Linaria* sect. *Supinae* terminals.

Haplotype networks

Prior to the haplotype network analysis, the ptDNA *Supinae* lineages were inferred by Bayesian Inference (BI), Maximum Likelihood (ML) and Maximum Parsimony (MP) analyses. To determine the optimal model of sequence evolution that best fits the sequence data (GTR+G for the three data sets), the AIC criterion was implemented in each data set using jModeltest 0.1.1 (Guindon & Gascuel 2003; Posada 2008). BI was conducted in MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003) via the CIPRES Science Gateway (http://www.phylo.org). We ran two sets of four Markov chains for 10000000 generations, sampling every 1000 generations. Parameters of both runs converged on the same stable distribution as seen in TRACER 1.4 (Rambaut 2007). Trees for the first 1000000 generations were discarded as burn-in. ML analysis was conducted using PhyML 3.0 (Guindon & Gascuel 2003; Guindon et al. 2010) including the model parameters previously obtained with jModeltest. Node supports for ML analysis were estimated using 500 non-parametric bootstrap replicates. MP analysis were run using the package TNT (Goloboff et al. 2008). An initial heuristic search was run using the Tree Bisection-

Reconnection (TBR) branch-swapping algorithm with 10000 replicates (saving two most-parsimonious trees per replicate). Trees obtained in the first search were used to start additional heuristics (Ratchet and Tree drifting algorithms). Bootstraping values were scored after resampling the matrix with 10000 standard replicates.

Breeding systems

To analyze relationships between P/O ratios and breeding systems in *Supinae* we additionally performed the Cruden's test (Cruden 1977) in 15 species, in 14 species to test reliability and in 1 species to have some information of the breeding system. For Cruden's test, ovules and pollen production per flower was quantified from three flower buds fixed in ethanol (70%) shortly before anthesis. The P/O ratios were obtained in three individuals per population and one population per species (see Table S5, Supporting Information) following Dafni (1992). The mean pollen-ovule ratios were classified into the five breeding systems of Cruden (1977).

Species traits associated with range expansion

For analyses in SIMMAP, morphological/standard priors were established following the approach of Schultz and Churchill (1999). Priors for each character were configured using beta distribution priors with 31 categories for the bias parameter and a gamma distribution prior for the overall evolutionary rate parameter with 90 categories. To determine values for α for the beta distribution prior, and α and β for the gamma distribution prior, we first ran a preliminary MCMC analysis under default settings. Samples from the posterior distribution of these parameters were used to obtain best-fitting distributions and parameter values for α and β using the R script available with SIMMAP. To account for phylogenetic uncertainty, character correlations were conducted in SIMMAP with the pruned MCC tree and 10 random trees from the stable posterior distribution of the *BEAST analysis. We conducted the analysis with 5 samples per tree, 5 prior draws for each character and 5 predictive samples to determine p-values for the correlation statistics *d* (differences between the observed and expected frequency of co-occurrence of states on the phylogeny) and *m* (fraction of the phylogeny one state is associated with another) (Huelsenbeck et al. 2003; see also http://www.simmap.com/pgs/stats_corr.html).

Species traits associated with diversification

The parameter values of the full BiSSE model were also analyzed in a Bayesian framework using a two-step process: first we analyzed parameter values using maximum likelihood and then used the obtained values as a prior for a MCMC sampling of parameters. This Bayesian approach (MCMC-BiSSE) provides a measure

of parameter uncertainty and, when repeated across a sample of plausible trees, can account for uncertainty in tree topology (Fitzjohn *et al.* 2009). The 10 randomly chosen trees were used to obtain the posterior distribution of parameter values. MCMC-BiSSE analyses were carried out with 10000 steps per tree (chain) and a prior for each parameter exponentially distributed (prioritizing small rates of change). After discarding the first 1000 steps of each chain as burn-in we summarized the results and plotted the obtained parameter values.

References

Blanco-Pastor JL, Vargas P, Pfeil BE (2012) Coalescent Simulations Reveal Hybridization and Incomplete Lineage Sorting in Mediterranean *Linaria*. *Plos One* **7**, e39089.

Cruden RW (1977) Pollen-Ovule Ratios: A Conservative Indicator of Breeding Systems in Flowering Plants. *Evolution* **31**, 32-46.

Dafni A (1992) *Pollination ecology: a practical approach.* Oxford University Press, USA.

Fitzjohn RG, Maddison WP, Otto SP (2009) Estimating trait-dependent speciation and extinction rates from incompletely resolved phylogenies. *Systematic Biology* **58**, 595-611.

Goloboff PA, Farris JS, Nixon KC (2008) TNT, a free program for phylogenetic analysis. *Cladistics* **24**, 774-786.

Guindon S, Dufayard JF, Lefort V*, et al.* (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Systematic Biology* **59**, 307-321.

Guindon S, Gascuel O (2003) A Simple, Fast, and Accurate Algorithm to Estimate Large Phylogenies by Maximum Likelihood. *Systematic Biology* **52**, 696-704.

Huelsenbeck JP, Nielsen R, Bollback JP (2003) Stochastic Mapping of Morphological Characters. *Systematic Biology* **52**, 131-158.

Librado P, Rozas J (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25**, 1451-1452.

Paradis E, Claude J, Strimmer K (2004) APE: analyses of phylogenetics and evolution in R language. *Bioinformatics* **20**, 289-290.

Posada D (2008) jModelTest: Phylogenetic Model Averaging. *Molecular biology and evolution* **25**, 1253-1256.

Rambaut A, Drummond, AJ (2007) Tracer v1.4, Available from BEAST Software website: http://beast.bio.ed.ac.uk/Tracer. Accessed November 2011.

Rambaut A, Drummond, AJ (2009) Tracer v1.5, Available from BEAST Software website: http://tree.bio.ed.ac.uk/software/tracer/. Accessed October 2012.

Ronquist F, Huelsenbeck JP (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**, 1572-1574.

Schultz TR, Churchill GA (1999) The Role of Subjectivity in Reconstructing Ancestral Character States: A Bayesian Approach to Unknown Rates, States, and Transformation Asymmetries. *Systematic Biology* **48**, 651-664.

Stephens M, Donnelly P (2003) A comparison of Bayesian methods for haplotype reconstruction from population genotype data. *The American Journal of Human Genetics* **73**, 1162-1169.

Stephens M, Smith NJ, Donnelly P (2001) A new statistical method for haplotype reconstruction from population data. *The American Journal of Human Genetics* **68**, 978-989.

Manuscript 4

Blanco-Pastor *et al.,* "Bee morphotypes explain floral variation in a radiation of *Linaria* species"

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Bee morphotypes explain floral variation in a radiation of *Linaria* species

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nectar spurs;
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Linaria;
long-tongued bees;
pollination.

Abstract

The role of pollinator shifts in floral divergence has long attracted the attention of evolutionary biologists. Although new results have been obtained recently, the effects of pollinators on plant species differentiation during rapid radiations, and the specific consequences of shifts within a single functional group of pollinators, are not well understood. Here, we evaluate pollinator-mediated flower-shape change in spurred flowers of closely related and recently diversifying *Linaria* species (sect. *Supinae* subsect. *Supinae*). We analysed the morphological features of *Linaria* flowers associated with the morphotypes of the predominant pollinators. First, the principal pollinators of *Linaria* species (long-tongued bees) were determined by extensive surveys. Then, the size and shape of flowers were analysed by means of standard and geometric morphometric measures. Standard measures failed to find relationships between the sizes of pollinators and flowers. However, geometric morphometric data and a discriminant-function analysis revealed that floral shapes with differential restrictiveness correlated with pollinator morphotypes. *Linaria* species with the most restrictive flowers displayed the most slender spurs and were pollinated by bees with larger proboscises. Restrictive flower shapes likely appeared more than once from a less restrictive ancestor during the evolutionary history of the study group. We show that floral variation can be driven by shifts between pollinators belonging to a single functional group, and that such transitions may have had important consequences for plant species differentiation during rapid radiations.

Introduction

The evolution of flower shape has been studied by several disciplines that seek to understand the basis of plant speciation, including developmental genetics, phylogeny, and evolutionary ecology (e.g. Cubas *et al.*, 1999; Ree & Donoghue, 1999; Gómez *et al.*, 2008). The remarkable variety of angiosperm flower shapes is thought to have evolved as a

consequence of strong pollinator-driven selection towards greater attractiveness and efficient pollen transfer (Darwin, 1862; Robertson, 1888; Stebbins, 1970; Faegri & Van der Pijl, 1979; Lehrer *et al.*, 1995; Møller, 1995; Wilson & Thomson, 1996; Endress, 1999; Anderson & Johnson, 2008). In the last decades, the relative importance of pollinator-mediated selection as a source of plant speciation has been challenged on the basis of apparent widespread generalisation in pollination systems (Herrera, 1996; Waser *et al.*, 1996; Johnson & Steiner, 2000; Strauss *et al.*, 2005), and the fact that floral phenotypes can evolve in response to selection from a combination of pollinators (Strauss *et al.*, 2005; Aigner, 2006; Herrera *et al.*, 2006). Regardless of the active debate on their role in plant speciation, pollinators are considered to play a major role in flowering plant differentiation through differential patterns of gene flow (Grant, 1949; Kay & Sargent, 2009; Yoder *et al.*, 2013). Flower specialisation may even occur in the most generalist plant species due to differences in the frequency of flower visits and differences in the pollinator effectiveness for particular plant species (e.g. Stebbins, 1970; Fulton & Hodges, 1999; Fenster *et al.*, 2004; Gómez *et al.*, 2008; Gómez *et al.*, 2013).

A vast amount of documentation can be found that describes the variation in flower forms associated with pollinator fauna (e.g. Armbruster, 1985; Robertson & Wyatt, 1990; Steiner & Whitehead, 1991; Fulton & Hodges, 1999; Schemske & Bradshaw, 1999; Castellanos *et al.*, 2003; Kephart & Theiss, 2004; Pérez-Barrales *et al.*, 2007; see Kay & Sargent, 2009 for review). Most of the phylogenetic studies of flower specialisation have focused on major flower-shape changes or morphological combinations (i.e., pollination syndromes), and the recognition of pollinators as functional groups defined by their ecological and morphological similarities (Faegri & Van der Pijl, 1979; Fenster *et al.*, 2004). Pollination syndromes have been studied extensively in the literature. But recently there has been a call for direct studies on the correlation between flower and pollinator features by analysis of specific traits, rather than studies based on evolutionary associations at larger scales (Galen & Cuba, 2001; Gómez *et al.*, 2006; Ollerton *et al.*, 2009; Smith, 2010; van der Niet *et al.*, 2013). Ecological patterns can only give insights into current (or very recent) differentiation processes. Therefore, it is difficult to infer the adaptive role of specific floral phenotypes from the existing interactions with pollinators (Herrera, 1996; Ollerton, 1996). Currently, there is a lack of species-level phylogenetic analyses combined with pollinator observations in recent plant radiations that consist of partially co-existing relatives (but see Johnson & Steiner, 1997; Valente *et al.*, 2012; van der Niet *et al.*, 2013). These kind of studies may provide important information on plant groups of recent differentiation that retain interfertility, for which floral isolation may be especially relevant (Kay & Sargent, 2009).

The genus *Linaria* constitutes the richest genus (~150 spp.) of the snapdragon tribe (Antirrhineae) (Sutton, 1988), and many species presumably originated from recent radiations (Blanco-Pastor & Vargas, 2013; Fernández-Mazuecos *et al.*, 2013). *Linaria* flowers display a complex spurred corolla that is potentially linked to pollinator specialisation. The flowers of *Linaria* have recently become a research focus in ecological interactions (Sánchez-Lafuente, 2007; Sánchez-Lafuente *et al.*, 2011) and developmental genetics of flower shape (Cubas *et al.*, 1999; Box *et al.*, 2011). Contrasting flower morphologies have been detected previously in *Linaria*, and were considered as an adaptation to divergent strategies of pollen placement on nectar-feeding insects (Robertson, 1888; Fernández-Mazuecos *et al.*, 2013). Morphological variation has been primarily associated with narrowing of the corolla tube (Viano, 1969; Sutton, 1988; Fernández-Mazuecos *et al.*, 2013), and is likely driven by selective forces of pollinator types such as bees versus dipterans/lepidopterans. In this genus, differences in per-visit effectiveness of pollinators of the same functional group (long-tongued bees) have also been documented (Sánchez-Lafuente et al., 2011), but detection of subtle floral divergence driven by such pollinators has not been addressed. Furthermore there are few reports in the literature of floral divergence from this kind of

transition (Johnson & Steiner, 1997; van der Niet et al., 2013).

Studies focused on fine-scale flower shape have been historically limited by the difficulties involved in the quantification of form (Rohlf, 1990). Flower shape has usually been studied as a qualitative trait or as a variable composed of several linear measurements (Neal *et al.*, 1998; Endress, 1999; Galen & Cuba, 2001). In the last decade, geometric morphometrics has emerged as a useful tool for the study of corolla-shape variation (Herrera, 1993; Gómez *et al.*, 2006; Gómez & Perfectti, 2010; Fernández-Mazuecos *et al.*, 2013). Here, we use geometric morphometrics to study fine-scale shape variation in flowers of closely related *Linaria* species that presumably originated in a recent radiation (Blanco-Pastor *et al.*, 2012; Blanco-Pastor *et al.*, 2013; Blanco-Pastor & Vargas, 2013). Specifically, we aim to achieve the following objectives: (i) obtain standard measurements of flowers (spur tip-stigma) and pollinators (proboscis tip-mesothorax) [midpoint of mesothorax (scutum) is the effective contact zone with floral stigma] to evaluate pollinator-flower morphological fitting or correlation; (ii) obtain geometric morphometric variables of flowers (relative warps, RWs); (iii) explore the functional significance of RWs that contribute most to explain pollinator-associated morphological variation; and (iv) reconstruct flower-shape variation during the history of these *Linaria* species.

Materials and Methods

Study species

We used the following 10 *Linaria* species belonging to subsect. *Supinae* of sect. *Supinae sensu* Blanco-Pastor *et al.* (2012) (hereafter subsect. *Supinae*), a monophyletic group of species inhabiting narrow ranges of southern Iberia: *L. aeruginea*, *L. almijarensis*, *L. amoi*, *L. anticaria*, *L. depauperata*, *L. lilacina*, *L. platycalyx*, *L. polygalifolia*, *L. tristis* and *L. verticillata* (Fig. 1). Because of their recent diversification in the Quaternary (Blanco-Pastor *et al.*, 2012; Blanco-Pastor *et al.*, 2013; Blanco-Pastor & Vargas, 2013), these species conform a suitable study group to test whether changes in ecological interactions have fostered differentiation of species. These species present the largest flowers within sect. *Supinae* (Sutton, 1988; Sáez & Bernal, 2009; Blanco-Pastor *et al.*, 2012). They display zygomorphic corollas with a cylindrical tube calcarate (spurred) abaxially at base and a palate (lower lip) occluding the mouth of the tube. The occluded personate form and consistency of the corolla requires the action of a strong or heavy insect to get access to pollen and nectar reward, which has long been considered as an adaptation to bee pollination (melittophily) (Müller, 1873; Hill, 1909; Sutton, 1988; Vargas *et al.*, 2010). The long spur of subsect. *Supinae* flowers debars short-tongued bees from sucking the nectar (Knuth, 1909; Sutton, 1988).

Flower visitor surveys

We counted and identified floral visitors in 1-3 populations per species. A total of 9609 minutes of observation evenly distributed among species were carried out during 5 years (2009-2013). Visits were considered legitimate when the insect opened the corolla tube and touched the anthers and stigma. We considered the pollinators with highest frequency of visits as the principal pollinators for each *Linaria* species because of their substantial percentage of visits (see below). As an estimate of the sampling effort for each *Linaria* species, we performed accumulation curves using the package *vegan* (Oksanen *et al.*, 2007) of R software (http://www.R-project.org).

Flower and pollinator sizes

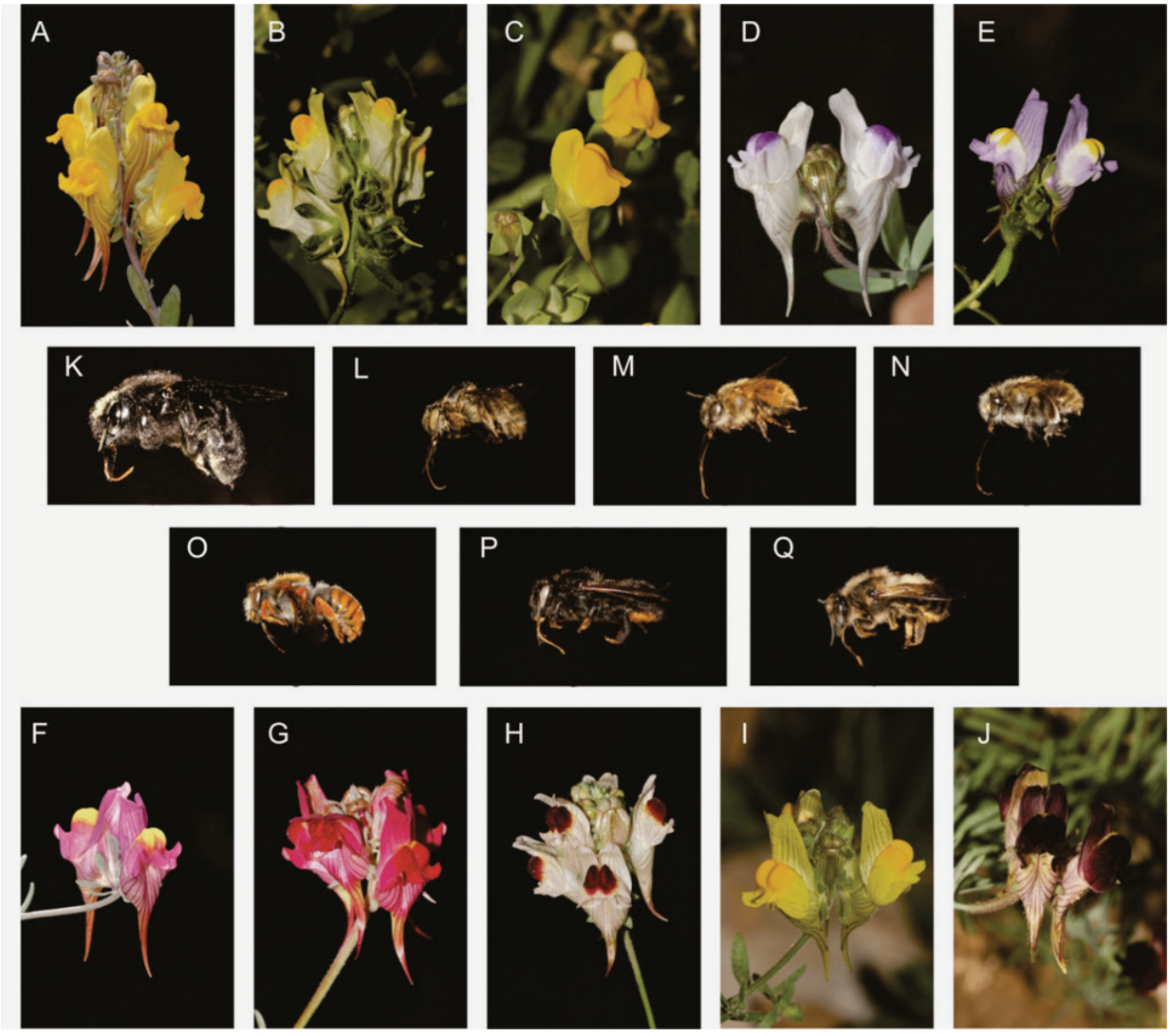
The complex corolla of *Linaria* can be separated into two major components: corolla tube and spur. We combined measurements of these two structures in order to characterize the inter- and intra-specific variability of the corolla size in subsect. *Supinae* species. One flower was randomly chosen in 25-36 individuals per species along 1-3 flowering seasons (a total of 286 flowers). Corolla length was scored in scaled digital photographs. Specifically, spur length was measured from the corolla-calyx insertion to the spur tip (Fig. 2A) and tube

length was measured from the corolla-calyx insertion to the stigma placed in the mouth of the tube (Fig 2A).

The *scutum* (midpoint of mesothorax) of bees is the typical contact zone with the stamens and the stigma during nototribic pollination (an effective mode of pollination in *Linaria*) (Kampny, 1995). Floral-visitor size was analysed as the distance between the extreme of the buccal apparatus and the *scutum*. For that, we combined two measures: (i) *scutum*-head and (ii) proboscis (Fig. 2B). The sum of both measures is hereafter called "pollinator contact length" (*PCL*). Measurements were taken in 6-21 individuals of the predominant flower visitors (a total of 152 individuals). For that we analysed scaled digital photographs with the software ImageJ 1.44p (Abràmoff *et al.*, 2004).

Flower shape

Flower shape was studied by means of geometric morphometric tools, using a landmark-based methodology that eliminates the effect of variation in the location, orientation, and scale of the specimens (Bookstein, 1997; Zelditch *et al.*, 2012). We took a digital photograph of one flower per individual in lateral view and planar position (286 flowers). We defined 4 coplanar landmarks and 13 semilandmarks located along the outline of the flowers which were considered satisfactory to define flower shape (Fig. 2A). Landmarks were captured at points of evident homology across species (Zelditch *et al.*, 2012): placement of upper stamens (landmark 1), the hinge of the lower lip (landmark 2) the spur tip (landmark 9) and at the corolla-calyx insertion



← **Fig. 1** – Photographs of *Linaria* sect. *Supinae* subsect. *Supinae* species: (A) *L. polygalifolia*, (B) *L. verticillata*, (C) *L. platycalyx*, (D) *L. anticaria*, (E) *L. lilacina*, (F) *L. depauperata*, (G) *L. amoi*, (H) *L. almijarensis*, (I) *L. aeruginea* and (J) *L. tristis*; and their most frequent visitors: (K) *Xylocopa violacea*, (L) *Anthophora crassipes*, (M) *Anthophora plagiata*, (N) *Anthophora plumipes*, (O) *Rhodanthidium sticticum*, (P) *Chalicodoma parietina* and (Q) *Chalicodoma pyrenaica*. Links among *Linaria* species and their predominant visitors: (A – K), (B – L), (C – M), (D and E – N), (F,G,H – O), (I – P), (J – Q).

(landmark 15) (Fig. 2A). We captured landmarks and semilandmarks using the software tpsDig 2.16 (Rohlf, 2010a). Three out of four landmarks were considered to be Type I (1,2 and 15) whereas landmark 9 may be considered Type II as it represents the tip of a structure and cannot be defined in term of other specific local features (Bookstein, 1997; Zelditch *et al.*, 2012). The semilandmarks were important for quantifying shape in the corolla that lack clear homologous points. These were placed by increments along the length of the curves typically equal but sometimes varying in order to reflect the complexity of the curve (Zelditch *et al.*, 2012). We used tpsRelw 1.49 (Rohlf, 2010b) to rotate, translate and scale landmark coordinates through generalized least squares superimposition (Bookstein *et al.*, 1985; Bookstein, 1997). Semilandmarks were slid along the corolla contours to minimize bending energy. This was done because the spacing of the semilandmarks was defined extrinsically. Thus, sliding to minimize the bending energy of the deformation adjusts the spacing of the semilandmarks to minimize the implication of semilandmanks placement on the detection of shape changes (Zelditch *et al.*, 2012). The software tpsRelw calculated, for each individual, a series of shape variables: uniform components and *partial warps* scores (non-uniform components). The principal components of the covariance matrix of the *partial warps* scores (*relative warps*, RW) were also obtained. We obtained $2p - 4$ (=30) orthogonal RWs (where p is the total number of landmarks and semilandmarks) that summarized shape differences among specimens (Zelditch *et al.*, 2012).

Statistical analyses

In order to investigate the size matching between flowers and insects we summarized their linear measures in box-plot graphs and examined the degree of overlap. The correlation between the mean *Linaria* corolla length and the mean *PCL* among species was explored using a Pearson's product-moment correlation test.

Flower visitors were classified in three taxonomic/morphological groups: (i) *Xylocopa violacea* (the longest bee species in europe), (ii) the genus *Anthophora* (species characterized by very long tongues); and (iii) the family Megachilidae (*Chalicodoma* spp. and *Rhodanthidium sticticum*). Pairwise one-way analysis of variance (ANOVA) was used to assess size differences among the three groups.

Corolla shape differences were assessed by means of a discriminant function analysis (DFA) that used RW scores as shape variables. The DFA was performed to reveal the most important characters contributing to the differentiation of individuals grouped by morphotypes of predominant pollinators (*Xylocopa*, *Anthophora* and *Megachilidae*, see below) and to test the assignment of specimens to predefined groups using two calculations: one computed with the original dataset and a more reliable cross-validation based approach in which the case that is being predicted is left out of the categorization process. The two discriminant functions of the DFA (the number of functions is g - 1, where g is the number of categories in the grouping variable) were represented in a scatterplot and the relative contribution of each variable (RW) in the discriminant function was explored. Statistical analyses were carried out in the SPSS 21.0 package (IBM Corp., Armonk, NY).

Flower shape evolution

We traced the evolution of pollinator shifts in subsect. *Supinae* by using ITS and AGT1 sequences previously obtained in Blanco-Pastor *et al.* (2013). Gene tree incongruence was found

among the two loci (see Blanco-Pastor *et al.*, 2013), therefore we performed independent ancestral state reconstructions (ASRs) in the two gene trees constructed with MrBayes 3.2 (Ronquist *et al.*, 2012). The unphased sequences of these two datasets were used. Additionally, we used the *BEAST species tree of Blanco-Pastor *et al.* (2013), that was constructed with phased sequences under the multispecies-coalescent model (Heled & Drummond, 2010), which accommodates phylogenetic incongruence assuming that it is exclusively caused by incomplete lineage sorting. Outgroup and *L. glacialis* terminals were pruned from the *BEAST tree because we failed to obtain pollinator data. To account for the topological uncertainty obtained in the Bayesian analyses of MrBayes and *BEAST, the reconstructions of pollinator shifts were conducted in 1000 trees from the stable posterior distribution of each analysis and summarized in the MrBayes 50% majority rule consensus trees and in the *BEAST maximum clade credibility tree.

In the phylogenies we mapped the three pollinator morphotypes which were linked to three flower morphotypes as moderately-to-strongly supported by the DFA analysis (89.5%/84.6% of correct classification, see Results). Ancestral states were reconstructed under parsimony in Mesquite 2.75 (Maddison & Maddison, 2011) using the "trace character over trees" option. The number of pollinator shifts was plotted using the "summarize state changes over trees" option.

Diversification rates

We estimated net diversification rates for subsect. *Supinae* in order to test for evolutionary radiation in subsect. *Supinae*. Diversification rates were compared with cases of exceptional plant radiations reported by previous authors (Valente *et al.*, 2010; Bell *et al.*, 2012). We used the method of Magallón and Sanderson (2001) implemented in the R package *geiger* (Harmon *et al.*, 2008) as similarly done by the authors mentioned above. We considered two extremes of the relative extinction rate (ε = 0, no extinction and ε = 0.9 high extinction rate, were ε = extinction rate/speciation rate). Species richness was estimated from the number of species that fell into subsect. *Supinae* clade (16) as obtained in a recent inclusive species tree analysis of sect. *Supinae* (Blanco-Pastor & Vargas, 2013). Diversification rates were calculated for crown and stem ages from the *BEAST analysis of Blanco-Pastor *et al.* (2013). Time estimates of Blanco-Pastor and Vargas (2013) were not used because they may be biased towards more recent divergence times as a consequence of the inclusion of plausible hybrid/introgressed species (Leaché *et al.*, 2013) (see Blanco-Pastor & Vargas, 2013).

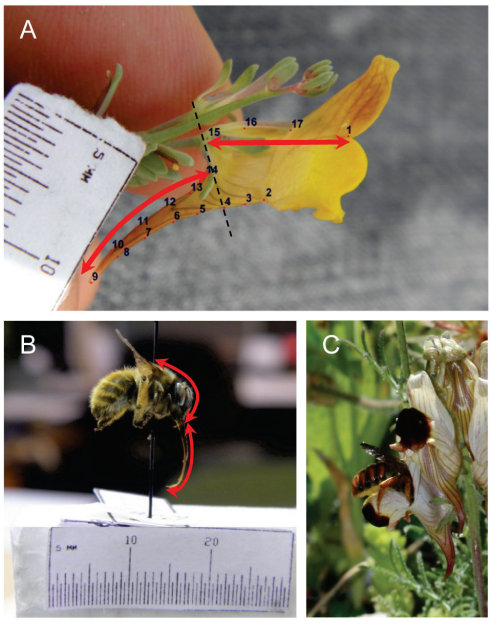


Fig. 2 – Measures used in morphometric analyses: (A) metric measures (spur and corolla tube length), landmarks (1, 2, 9, 15) and semilandmarks (3-8, 10-14, 16-17) of flowers; and (B) metric measures [*scutum*-head and proboscis, pollinator contact lenght (*PCL*)] of pollinators. (C) Example of a legitimate visit in a *Linaria* flower (*Rhodanthidium sticticum* visiting *L. almijarensis*).

Table 1. Pollinator censuses data including census time, pollinators observed, number of flowers visited and percentage of visits of each pollinator.

Linaria species	Locality	Census time in minutes (year of census)	Pollinators observed	No. of flower visits	% visits
Linaria aeruginea (Gouan) Cav.	Spain. Granada. Sierra Nevada, Hoya de la Mora	270 (2009)	*Chalicodoma parietina* (Geoffroy, 1785)*	283	79.94
	Spain. Granada. Sierra Nevada, Estación Borreguiles	465 (2010)	*Chalicodoma pyrenaica* Lepeletier, 1841	30	8.47
	Spain. Granada. Sierra Nevada, Las Sabinas	480 (2010)	*Hoplitis praestans* (Morawitz, 1893)	16	4.52
			Osmia gallarum Spinola, 1808	13	3.67
			Osmia parietina Curtis, 1828	7	1.98
			Megachile albonotata Radoszkowski, 1886	5	1.41
Linaria almijarensis Campo & Amo	Spain. Jaén. Cabra	520 (2010); 583 (2012)	*Rhodanthidium sticticum* (Fabricius, 1787)*	246	44.40
			Anthophora atroalba Lepeletier, 1841	115	20.76
			Apis mellifera iberiensis Engel, 1999	94	16.97
			Anthophora plagiata (Illiger, 1806)	69	12.45
			Chalicodoma pyrenaica Lepeletier, 1841	25	4.51
			Lasioglossum buccale (Pérez, 1903)	3	0.54
			Anthidium manicatum (Linnaeus, 1758)	2	0.36
Linaria amoi Campo ex Amo	Spain. Málaga. Sierra Tejeda, Canillas de Aceituno	135 (2010); 305 (2011); 150 (2013)	*Rhodanthidium sticticum* (Fabricius, 1787)*	22	84.62
			Osmia andrenoides Spinola, 1808	3	11.54
			Osmia submicans Morawitz, 1870	1	3.85
Linaria anticaria Boiss. & Reut.	Spain. Málaga. Torcal de Antequera	487 (2010); 437 (2012)	*Anthophora plumipes* (Pallas, 1772)*	261	40.03
			Osmia andrenoides Spinola, 1808	242	37.12
			Rhodanthidium sticticum (Fabricius, 1787)	65	9.97
			Ceratina cucurbitina (Rossi, 1792)	54	8.28
			Macroglossum stellatarum Linnaeus, 1758	22	3.37
			Ceratina dentiventris (Gerstäcker,	6	0.92

			1869)		
			cf. *Anthidium sp.*	2	0.31
Linaria depauperata Leresche ex Lange	Spain. Alicante. Petrel	142 (2010); 1491 (2013)	*Rhodanthidium sticticum* (Fabricius, 1787)*	32	91.43
			cf. *Anthophora sp.*	3	8.57
Linaria platycalyx Boiss.	Spain. Cádiz. Sierra de Grazalema, Zahara de la Sierra	610 (2010); 438 (2012)	*Osmia andrenoides* Spinola, 1808	40	30.53
			Anthophora plagiata (Illiger, 1806)*	39	29.77
			Ceratina cucurbitina (Rossi, 1792)	39	29.77
			Chalicodoma pyrenaica Lepeletier, 1841	5	3.82
			Osmia caerulescens (Linnaeus, 1758)	5	3.82
			Rhodanthidium sticticum (Fabricius, 1787)	2	1.53
			Lasioglossum bucale (Pérez, 1903)	1	0.76
Linaria polygalifolia Hoffmanns. & Link.	Spain. Portugal. Algarve. Monte Gordo	737 (2013)	*Xylocopa violacea* (Linnaeus, 1758)*	249	96.14
			Osmia rufohirta Latreille, 1811	4	1.54
			Rhodanthidium sticticum (Fabricius, 1787)	3	1.16
			Lasioglossum albovirens (Pérez, 1895)	3	1.16
Linaria tristis (L.) Mill.	Spain. Cádiz. Sierra de los Alcornocales, Alcalá de los Gazules	468 (2010); 525 (2012); 375 (2013)	*Chalicodoma pyrenaica* Lepeletier, 1841*	29	70.73
			Anthophora plagiata (Illiger, 1806)	10	24.39
			Ceratina cucurbitina (Rossi, 1792)	2	4.88
Linaria verticillata Boiss.	Spain. Granada. Sierra Nevada, Las Sabinas	491 (2010)	*Anthophora crassipes* Lepeletier, 1841*	53	84.13
	Spain. Granada. Sierra Nevada, Los Peñones	500 (2012)	*Osmia emarginata* Lepeletier, 1841	10	15.87

*Principal pollinator as considered in this study.

†The principal pollinator of *L. lilacina* is *Anthophora plumipes* (Syn. *A. acervorum*) (Sánchez-Lafuente *et al.*, 2011)

Results

Pollinator surveys

A low number of insect species (2-7) were found visiting the flowers of *Linaria* species and all were bees (except for the hawk-moth *Macroglossum stellatarum* that visited *L. anticaria* flowers). In all *Linaria* species except *L. platycalyx* (see Table 1) we found a single principal visitor with a percentage of visits ranging from 40.03 to 96.14 (Table 1, Fig. 1). Sampling effort was considered adequate as all species' accumulation curves became nearly asymptotic at the last stages of the sampling (Fig. S1, Supporting Information).

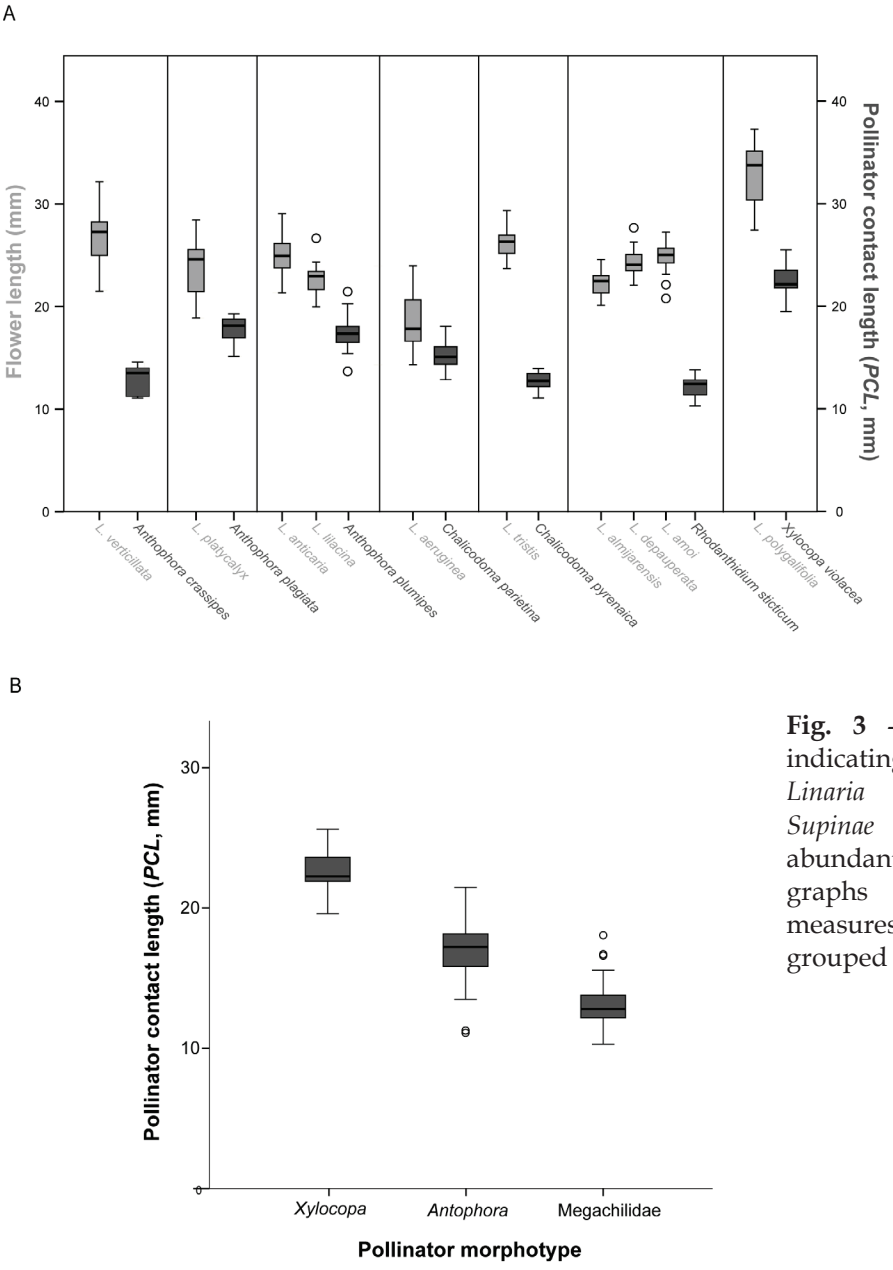


Fig. 3 – (A) Box plot graphs indicating linear measures of *Linaria* sect. *Supinae* subsect. *Supinae* species and their most abundant pollinators. (B) Box plot graphs representing linear measures of most frequent visitors grouped in three morphotypes.

Flower and pollinator sizes

Flower and pollinator sizes were found to be rather variable. Flower sizes (spur + tube) ranged from ca. 15 to 35 mm and pollinator sizes (*PCLs*) ranged from ca. 10 to 25 mm (Fig. 3A, Table S1 and S2, Supporting Information). *Linaria* flowers were shown to be larger than the *PCL* of the principal pollinators in all species. In all ten cases there was no overlap

between the interquartile ranges of the flower sizes and the *PCLs* of their principal pollinators (Fig. 3A). Mean values for flower length of *Linaria* species and *PCLs* of the principal pollinators were not significantly correlated (r = 0.472; P = 0.168; Fig. 4). Interquartile of pollinator group sizes (*Xylocopa*, *Anthophora*, Megachilidae) showed no overlap between the box plot graphs (Fig. 3B). The pairwise one-way ANOVAs indicated significant size differences among groups ($F_{\text{Anthophora-Megachilidae}}$ = 239.229, P < 0.001; $F_{\text{Xylocopa-Anthophora}}$ = 96.186, P < 0.001; $F_{\text{Megachilidae-Xylocopa}}$ = 723.603, P < 0.001).

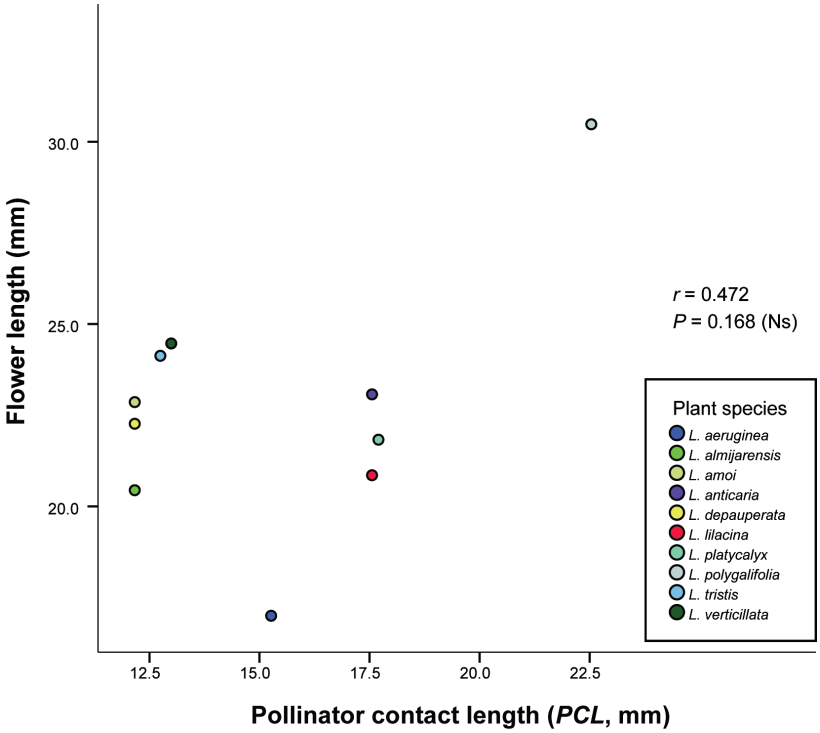


Fig. 4 – Scatter plot of mean flower length versus mean pollinator length measured in 25-36 individuals per *Linaria* species (286 individuals) and 6-21 individuals per principal visitor species (152 individididuals). Pearson's product-moment correlation coefficient and its statistical significance value are indicated.

Flower shape differences

The DFA revealed statistical significance for the three *Linaria* groups predefined by the predominant pollinator morphotype (Wilks' lambda = 0.185, P < 0.001), with 89.5% of original grouped cases correctly classified and 84.6% of cross-validated grouped cases correctly classified (Fig. 5, Table 2). The first function of the DFA discriminated between the specimens pollinated by Megachilidae bees and the other two groups, while the second function discriminated specimens pollinated by *Xylocopa violacea* and the other two groups (Fig. 5). The shape variables (RWs) that contributed most to the discriminant functions were associated to the relative spur width (RW 12; discriminant function 1) and relative spur length (RW 2; discriminant function 2) (Values of discriminant function coefficients (RWs) are given in Fig. S2, Supporting Information).

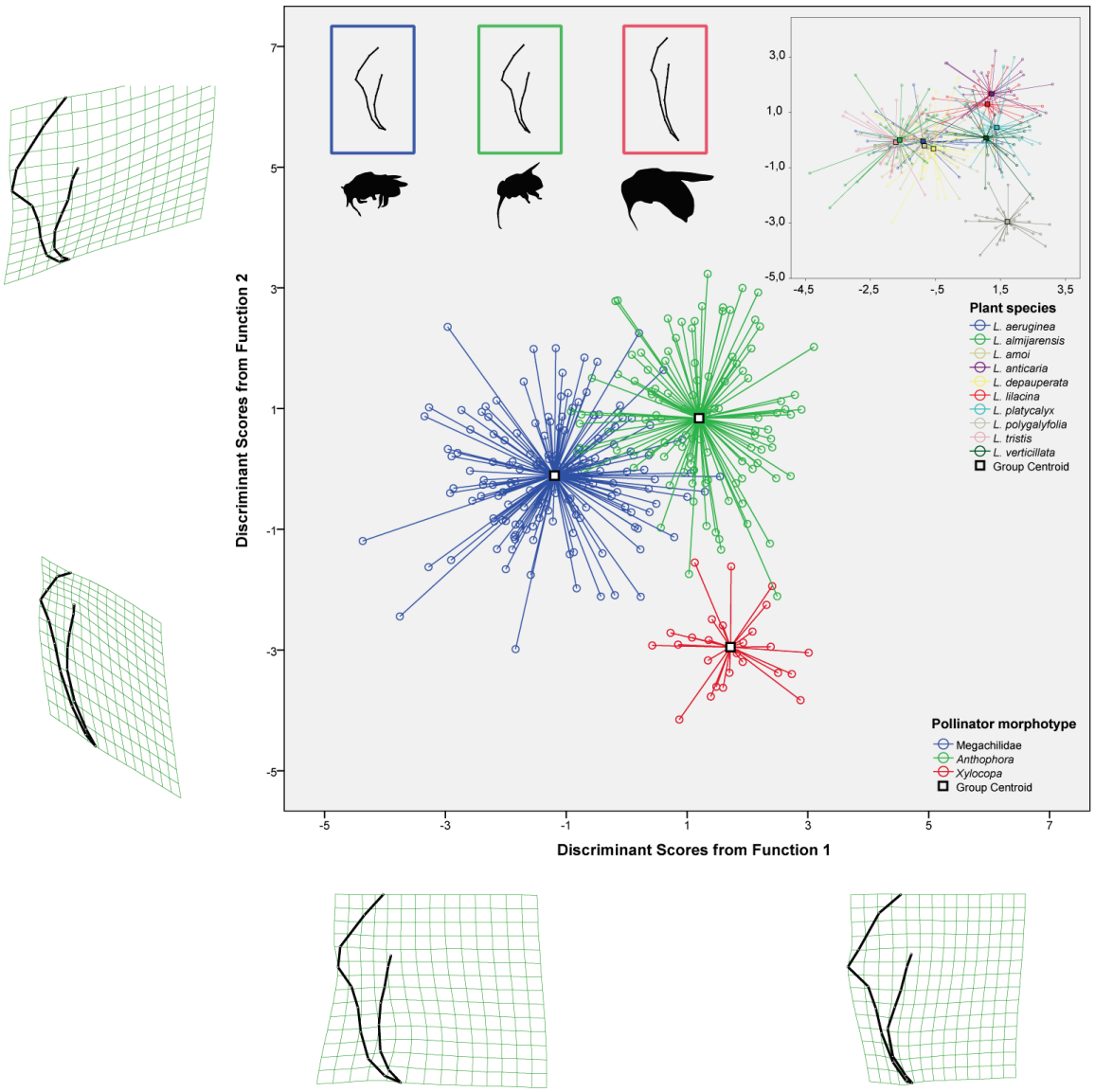
Evolution of flower shape

The ancestral state reconstruction (ASR) under parsimony performed in ITS and AGT1 gene trees recovered a Megachilidae flower type as ancestral to subsect. *Supinae* (Fig. 6A-B). Number of shifts between *Anthophora* and Megachilidae flower types differed in the two analyses. The ITS trees recovered two shifts from *Anthophora*-type to Megachilidae-type flowers with the highest frequency (0.71) and one shift in the opposite direction (0.71) (Fig. 6A, Table 3). The AGT1 trees recovered zero shifts from *Anthophora*-type to Megachilidae-type with highest frequency (0.72) and three shifts in the opposite direction (0.70) (Fig. 6B,

Table 3). The ASR performed in the *BEAST trees also recovered a Megachilidae flower type as ancestral to subsect. *Supinae* (Fig. 6C). The number of shifts recovered in the *BEAST tree was uncertain but showed a lower frequency of shifts from *Anthophora*-type to Megachilidae-type [zero shifts recovered with highest frequency (0.45)] than in the opposite direction [three shifts recovered with highest frequency (0.33)] (Fig. 6C, Table 3). The ITS, AGT1 and *BEAST trees recovered one shift from Megachilidae-type to *Xylocopa*-type flowers with the highest frequency (0.94, 0.87 and 0.83, respectively) and zero shifts in the remaining transitions involving Xylocopa-type flowers with frequencies above 0.86 (Table 3).

Diversification rates

We estimated the diversification rates of subsect. *Supinae* to be 0.36-1.82 species per million years (sp/Ma) and 1.10-5.52 sp/Ma for the stem group assuming high extinction and no extinction respectively. Diversification rates for the crown group was estimated to be 0.48-2.52 sp/Ma and 1.16-6.09 sp/Ma assuming high extinction and no extinction respectively. Overall, net diversification rate of subsect. *Supinae* ranged from 0.36 to 6.09 sp/Ma (Table 4). These estimates resembled those of the most rapid plant radiations reported to date (reviewed in Valente *et al.*, 2010; and Bell *et al.*, 2012).



← **Fig. 5** – Discriminant function analysis (DFA) of geometric morphometric variables (relative warps, RWs). Analysis was performed by using pollinator morphotype as grouping variable. Upper left inset: consensus flower shape of individuals grouped by pollinator morphotype. Upper right inset: DFA with colours representing *Linaria* species. The extreme values of RWs with highest weightings in the two discriminant functions are represented in the axes (see also Fig. S2).

Table 2. Predicted group membership obtained in the DFA analysis. The numbers going down each column indicate the number (count) and percentage (%) of *Linaria* flowers that were correctly/incorrectly classified into the three pollinator groupings.

		Predicted Group Membership			
	Pollinator	Megachilidae	*Anthophora*	*Xylocopa*	Total
Original count (%)	Megachilidae	134 (89.9)	15 (10.1)	0 (0)	149 (100)
	Anthophora	13 (11.7)	96 (86.5)	2 (1.8)	111 (100)
	Xylocopa	0 (0)	0 (0)	26 (100)	26 (100)
Cross-validated* count (%)	Megachilidae	125 (83.9)	23 (15.4)	1 (0.7)	149 (100)
	Anthophora	14 (12.6)	92 (82.9)	5 (4.5)	111 (100)
	Xylocopa	0 (0)	1 (3.8)	25 (96.2)	26 (100)

*In the cross-validation procedure the case that is being predicted is left out of the categorization process.
†89,5% of original grouped cases correctly classified.; 84,6% of cross-validated grouped cases correctly classified.
‡Statistical significance for the groups predefined by the principal pollinator type: (Wilks' lambda = 0.185, P < 0.001)

Discussion

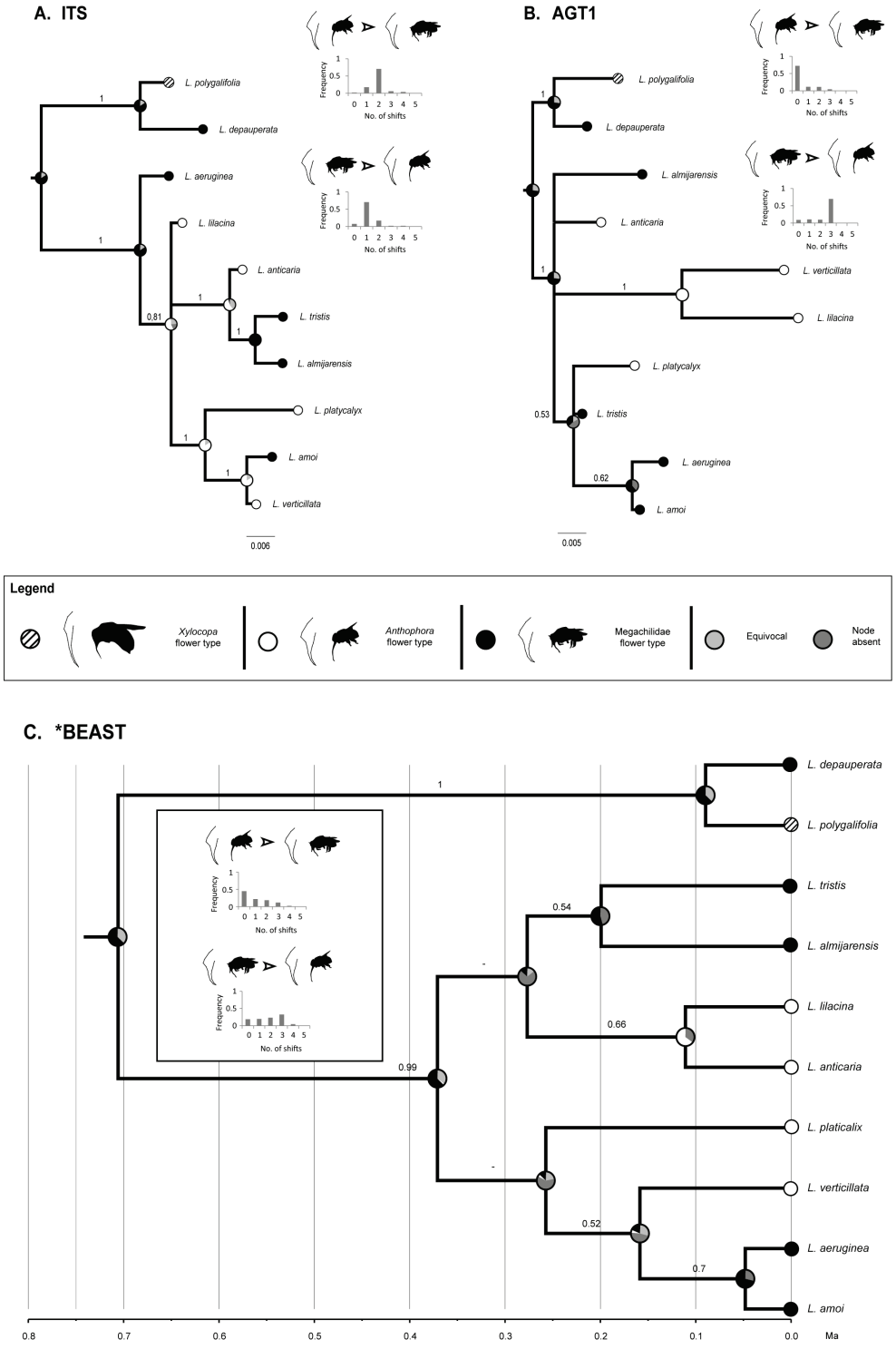
Restrictive flowers and specialised visitors

The role of nectar spurs in plant specialisation and flower restriction has been addressed in many studies during the last two decades (Robertson & Wyatt, 1990; Hodges & Arnold, 1994, 1995; Hodges, 1997; Johnson & Steiner, 1997; Fulton & Hodges, 1999; Whittall & Hodges, 2007). The current study complements the existing literature on the association of spurred flower morphological changes with their principal pollinators. The approach taken here was novel, because nectar spurs were considered as multidimensional traits and were defined by several shape variables (measured as RWs) instead of single variables such as spur length, which was typically measured in previous studies. The flower sizes of closely related *Linaria* species analysed here showed neither fit nor correlation with the sizes of the principal pollinators (Figs. 3A and 4). By contrast, flower-shape differences according to pollinator morphotypes were supported in the DFA (Table 2 and Fig. 5).

Despite the incongruence displayed in the gene trees and the uncertainty obtained in the species tree, it seems that phylogenetic constraints have not played an important role in shaping floral morphology and pollinator preferences in subsect. *Supinae*, as shown by the evolution of similar flower morphologies in independent lineages (Fig. 6). This result is consistent with a recent study of sect. *Versicolores* (Fernández-Mazuecos *et al.*, 2013), in which two main types of *Linaria* flowers differing in tube width were associated with divergent strategies of pollen placement on nectar-feeding insects (*scutum* versus proboscis). All species analysed here displayed the typical broad-tubed morphology associated with nototribic pollination (Kampny, 1995), which is found in most *Linaria* species of other sections (Type I of sect. Versicolores, Fernández-Mazuecos *et al.*, 2013). Even though subsect. *Supinae* species displayed a similar morphology and were primarily visited by similar insects (long-tongued bees) performing the same nototribic strategy, we found differences in flower shape that correlated with the spur shape.

The main difference of *Xylocopa*-type flowers was their size compared with those of Megachilidae-type or *Antophora*-type flowers (Figs. 3A and 4). Because *Xylocopa* bees visited

only one species (*L. polygalifolia*), we focused on the shape differences between Megachilidae-type and *Antophora*-type flowers. The most restrictive flowers, which were characterised by narrow spurs, were found in populations pollinated primarily by *Anthophora* bees. Spur-shape changes might be linked to restriction of the access to nectar reward for certain pollinators, and provide insights for particular cases of subtle floral specialisation among spurred flowers that has rarely been described. Reward accessibility can affect the attraction of pollinators (Hodges & Arnold, 1995; Armbruster & Muchhala, 2009). Therefore, a



← **Fig. 6 –** Phylogenetic analyses and ancestral state reconstructions of flower types. Reconstructions performed in the 50% majority rule consensus tree of the (A) ITS and (B) AGT1 MrBayes analyses with nodes with PP<0.5 collapsed. (C) Ancestral state reconstruction performed in the dated MCC *BEAST species tree of Blanco-Pastor *et al.* (2013). The summary distributions of the number of shifts between flower types were inferred by parsimony optimization over 1000 trees from the posterior distribution of the Bayesian analyses.

Table 3. Summary frequencies of the number of shifts between flower types. Frequencies were inferred by parsimony optimization over 1000 trees from the posterior distribution of the MrBayes (ITS and AGT1) and *BEAST analyses.

		Frequency		
Flower shape shift	No. of shifts	ITS	AGT1	*BEAST
Anthophora-type to Megachilidae-type	0	0.02	0.72	0.45
	1	0.18	0.12	0.22
	2	0.71	0.11	0.19
	3	0.06	0.05	0.12
	4	0.04	0.00	0.03
Megachilidae-type to *Anthophora*-type	0	0.08	0.09	0.19
	1	0.71	0.11	0.20
	2	0.17	0.10	0.23
	3	0.02	0.70	0.33
	4	0.02	0.00	0.05
Anthophora-type to *Xylocopa*-type	0	0.95	0.87	0.89
	1	0.05	0.13	0.11
	2	0	0	0
	3	0	0	0
	4	0	0	0
Xylocopa-type to *Anthophora*-type	0	100	100	0.94
	1	0	0	0.06
	2	0	0	0
	3	0	0	0
	4	0	0	0
Megachilidae-type to *Xylocopa*-type	0	0.05	0.13	0.17
	1	0.95	0.87	0.83
	2	0	0	0
	3	0	0	0
	4	0	0	0
Xylocopa -type to *Megachilidae* -type	0	0.97	0.93	0.89
	1	0.03	0.07	0.11
	2	0	0	0
	3	0	0	0
	4	0	0	0

morphological change towards more restrictive spurs may have reduced visitation by other bees with shorter proboscises. Long-tongued *Anthophora* bees usually perform more crossing movements than other bees, therefore other bees with shorter tongues could be considered as pollen-wasting 'parasite' pollinators (sensu Thomson & Wilson, 2008) for these self-incompatible species (Blanco-Pastor & Vargas, 2013). This was evidenced by Sánchez-Lafuente *et al.* (2011), who reported a higher per-visit effectiveness of *Anthophora* bees when compared with that of *Apis* and *Bombus* bees due to a higher per-interaction effect associated with allogamous pollination in *Linaria lilacina* (subsect. *Supinae*).

In light of the expectedly higher per-visit effectiveness of *Antophora*, it is possible that the Megachilidae visitors might not be the principal pollinators of *L. depauperata*, *L. tristis* and *L. almijarensis*. Nevertheless, we considered that

our approach was appropriate because Sánchez-Lafuente *et al.* (2011) reported that a critical interaction-frequency ratio of 2.66 for *Apis*-*Anthophora* is required to consider Apis as the principal pollinator. In our study, we found only one case below this critical value (Megachilidae-*Anthophora* interaction-frequency ratio of 1.48 in *Linaria almijarensis*). Indeed, Megachilidae bees performed notably more allogamous visits compared to those of *Apis* (Blanco-Pastor *et al.*, pers. observ.).

The second-most frequent visitor of *L. platycalyx* (*Anthophora plagiata*) was considered the principal pollinator because of the remarkably higher per-interaction effect [higher pollen removal and allogamous visits compared with those of small bees, the other two most frequent pollinators (see Table 1)] (Blanco-Pastor *et al.*, personal observation).

Have pollinators driven differentiation in *Linaria*?

The *Linaria* species studied here should be considered as corolla specialists. Although flower specialisation may have played an important role in *Linaria* differentiation, it remains unclear whether flower shape shifts have promoted rapid speciation or whether other factors were also involved. Radiations in many plant groups have been historically thought to be a consequence of adaptive shifts between specialised pollination systems (Stebbins, 1970; Nilsson, 1988). Morphological differences in flower traits, such as nectar spurs, could theoretically promote floral isolation and speciation because their variation may reduce or enhance the effectiveness of pollen transfer by different types of pollinators (Nilsson, 1988; Robertson & Wyatt, 1990; Hodges & Arnold, 1995; Hodges, 1997; Johnson & Steiner, 1997; Fulton & Hodges, 1999). Additionally, variation in pollination effectiveness within a functional group of pollinators has been documented previously in subsect. *Supinae* (long-tongued bees, Sánchez-Lafuente *et al.*, 2011) and other angiosperms (Hymenoptera, Schemske & Horvitz, 1984; Lepidoptera, Pettersson, 1991; Diptera, Johnson & Steiner, 1997). Nevertheless, floral divergence is rarely sufficient to cause speciation on its own (Grant, 1949, 1981; Hodges & Arnold, 1994; Kay & Sargent, 2009; Valente *et al.*, 2012; Armbruster *et al.*, 2013).

The current ranges of subsect. *Supinae* species are mountains and coastal zones of southern Iberia, and their distributions have low overlap (see species' ranges in Blanco-Pastor & Vargas, 2013). Therefore, the rapid differentiation of this *Linaria* group may have been driven by allopatric speciation by means of ecogeographical barriers (c.f. van der Niet *et al.*, 2006; Schnitzler *et al.*, 2011). In that sense, sympatric speciation (initial-Reproductive Isolation model sensu Armbruster & Muchhala, 2009) may not account for the species diversity found in subsect. *Supinae.* This is true because flower shape changes were not consistent enough to exclude co-occurring bee morphotypes (see Table 1), therefore allowing gene flow among incipient species. Divergent selection in allopatry driven by geographical differences in pollinator fauna (Grant, 1949; Herrera *et al.*, 2006; Gómez *et al.*, 2008) may have been the mayor factor that explains flower variation in sect. *Supinae*. Additionally, upon secondary contact, flower specialization in may have favored divergence among incipient species by means of reinforcement of pre-existing reproductive isolation among these interfertile species (Reinforcement model) (Dobzhansky, 1937; Grant, 1949; van der Niet *et al.*, 2006; Armbruster & Muchhala, 2009).

Tracing morphological evolution in plant radiations

The estimated rates of diversification for subsect. *Supinae* are 0.36–5.52 (stem group sp/Ma) and 0.48–6.09 (crown group sp/Ma), which account for uncertainty in diversification times and extinction rates. These rates surpass most of the rapid radiations documented in plants (reviewed in Valente *et al.*, 2010; and Bell *et al.*, 2012), and provide one more example of a diversification burst in the Mediterranean.

The study of morphological evolution of incipient plant radiations is especially relevant because it may provide important insights into floral differentiation and isolation among species that retain interfertility

Table 4. Diversification rates of *Linaria* sect. *Supinae* subsect *Supinae* based on median crown and stem ages obtained in the *BEAST analysis of (Blanco-Pastor *et al.*, 2013). Estimates based on 95% highest posterior density are indicated in brackets.

	Diversification rate (stem age, sp/Ma)		Diversification rate (crown age, sp/Ma)	
	ε = 0.9	ε = 0	ε = 0.9	ε = 0
Linaria* sect. *Supinae* subsect. *Supinae	0.67 (0.36-1.82)	2.04 (1.10-5.52)	0.97 (0.48-2.52)	2.34 (1.16-6.09)

* ε, extinction rate as a fraction of speciation rate

(Kay & Sargent, 2009). Our study traced morphological transitions in a plant radiation with a recent origin in the Pleistocene. Evolutionary reconstructions of recent radiations have the advantages of population-based approaches, in which incipient differentiation enables the linking of current pollinator fauna with morphological transitions (Gómez *et al.*, 2013). Additionally, the study of radiations has the advantages of species-based approaches, which provides species relationships in a tree-like manner, and facilitates reconstruction of morphological transitions using phylogenetic methods (Nunn, 2011). Nevertheless, we should be cautious with interpretation of plant radiation phylogenetic reconstructions such as this of subsect. *Supinae*. First, a common problem may be a lack of phylogenetic resolution (Fig. 6C), which may be a consequence of low differentiation among early divergent species (see also Valente *et al.*, 2010; Bell *et al.*, 2012). Second, reticulate processes affecting phylogenetic reconstructions such as hybridisation or introgression (as previously evidenced in sect. Supinae, Blanco-Pastor *et al.*, 2012) may also be recurrent in plant radiations (Seehausen, 2004), and could hinder more complex scenarios of morphological evolution.

Population-based approaches account for intraspecific processes of morphological differentiation, which are recurrent in nature (Pérez-Barrales *et al.*, 2007; Anderson & Johnson, 2008). Therefore, as a complement to this study, further analyses at the population level will be valuable to validate the current hypothesis: that a similar restrictive flower shape has evolved several times in sect. *Supinae* as a barrier against less-efficient pollinators. High-throughput sequencing data (e.g. Baird *et al.*, 2008; Elshire *et al.*, 2011) and future development of analytical methods that account for the shared common ancestry and exchange of migrants between populations (Stone *et al.*, 2011) would greatly contribute to a better understanding of the evolutionary processes occurring in this group.

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Supporting Information

Supplementary Table 1 - Morphometric variables of *Linaria* specimens
Supplementary Table 2 - Morphometric data of pollinator specimens

Fig. S1 - Plot of cumulative species richness against sampling effort. Asymptotic curves indicate appropriate sampling effort in pollination censuses.

Fig. S2 - Standardized canonical discriminant function coefficients of the discriminant function analysis (DFA). The magnitudes of these coefficients indicate the effect of variables (relative warps, RWs) in the discriminant functions. Extreme values of RWs with highest scores are drawn.

References

Abràmoff, M. D., Magalhães, P. J. & Ram, S. J. 2004. Image processing with ImageJ. *Biophotonics international* **11**: 36-42.

Aigner, P. A. (2006) The evolution of specialized floral phenotypes in a fine-grained pollination environment. In: *Plant-Pollinator Interactions: From Specialization to Generalization*, (Waser, N. M. & Ollerton, J., eds.). pp. University of Chicago Press.

Anderson, B. & Johnson, S. D. 2008. The geographical mosaic of coevolution in a plant-pollinator mutualism. *Evolution* **62**: 220-225.

Armbruster, W. S. 1985. Patterns of character divergence and the evolution of reproductive ecotypes of *Dalechampia scandens* (Euphorbiaceae). *Evolution* **39**: 733-752.

Armbruster, W. S. & Muchhala, N. 2009. Associations between floral specialization and species diversity: cause, effect, or correlation? *Evolutionary Ecology* **23**: 159-179.

Armbruster, W. S., Shi, X.-Q. & Huang, S.-Q. 2013. Do specialized flowers promote reproductive isolation? Realized pollination accuracy of three sympatric *Pedicularis* species. *Annals of botany*: doi: 10.1093/aob/mct187.

Baird, N. A., Etter, P. D., Atwood, T. S., Currey, M. C., Shiver, A. L., Lewis, Z. A., Selker, E. U., Cresko, W. A. & Johnson, E. A. 2008. Rapid SNP discovery and genetic mapping using sequenced RAD markers. *PloS one* **3**: e3376.

Bell, C. D., Mavrodiev, E., Soltis, P., Calaminus, A., Albach, D., Cellinese, N., Garcia-Jacas, N. & Soltis, D. 2012. Rapid diversification of *Tragopogon* and ecological associates in Eurasia. *Journal of evolutionary biology* **25**: 2470-2480.

Blanco-Pastor, J. L., Fernández-Mazuecos, M. & Vargas, P. 2013. Past and future demographic dynamics of alpine species: limited genetic consequences despite dramatic range contraction in a plant from the Spanish Sierra Nevada. *Molecular Ecology* **22**: 4177-4195.

Blanco-Pastor, J. L. & Vargas, P. 2013. Autecological traits determined two evolutionary strategies in Mediterranean plants during the Quaternary: low differentiation and range expansion versus geographical speciation in *Linaria*. *Molecular Ecology* **22**: 5651–5668.

Blanco-Pastor, J. L., Vargas, P. & Pfeil, B. E. 2012. Coalescent Simulations Reveal Hybridization and Incomplete Lineage Sorting in Mediterranean *Linaria*. *PLoS ONE* **7**: e39089.

Bookstein, F. L. 1997. *Morphometric tools for landmark data: geometry and biology*. Cambridge University Press.

Bookstein, F. L., Chernoff, B., Elder, R. L., Humphries, J., Smith, G. R. & Strauss, R. E. 1985. *Morphometrics in evolutionary biology: the geometry of size and shape change, with examples from fishes*. Academy of Natural Sciences of Philadelphia Philadelphia.

Box, M. S., Dodsworth, S., Rudall, P. J., Bateman, R. M. & Glover, B. J. 2011. Characterization of *Linaria* KNOX genes suggests a role in petal-spur development. *The Plant Journal* **68**: 703-714.

Castellanos, M. C., Wilson, P. & Thomson, J. D. 2003. Pollen transfer by hummingbirds and bumblebees, and the divergence of pollination modes in *Penstemon*. *Evolution* **57**: 2742-2752.

Cubas, P., Vincent, C. & Coen, E. 1999. An epigenetic mutation responsible for natural variation in floral symmetry. *Nature* **401**: 157-161.

Darwin, C. 1862. *On the various contrivances by which British and foreign orchids are fertilised by insects: and on the good effects of intercrossing*. London: J. Murray.

Dobzhansky, T. 1937. *Genetics and the Origin of Species*. Columbia University Press, New York.

Elshire, R. J., Glaubitz, J. C., Sun, Q., Poland, J. A., Kawamoto, K., Buckler, E. S. & Mitchell, S. E. 2011. A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. *PloS one* **6**: e19379.

Endress, P. K. 1999. Symmetry in Flowers: Diversity and Evolution. *International Journal of Plant Sciences* **160**: S3-S23.

Faegri, K. & Van der Pijl, L. 1979. The principles of pollination ecology, 3rd rev. edn. *Pergamon Press, Oxford*.

Fenster, C. B., Armbruster, W. S., Wilson, P., Dudash, M. R. & Thomson, J. D. 2004. Pollination syndromes and floral

specialization *Annual Review of Ecology, Evolution, and Systematics* **35**: 375-403.

Fernández-Mazuecos, M., Blanco-Pastor, J. L., Gómez, J. M. & Vargas, P. 2013. Corolla morphology influences diversification rates in bifid toadflaxes (*Linaria* sect. *Versicolores*). *Annals of Botany* **112**: 1705-1722.

Fulton, M. & Hodges, S. A. 1999. Floral isolation between *Aquilegia formosa* and *Aquilegia pubescens*. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **266**: 2247-2252.

Galen, C. & Cuba, J. 2001. Down the tube: pollinators, predators, and the evolution of flower shape in the alpine skypilot, *Polemonium viscosum*. *Evolution* **55**: 1963-1971.

Gómez, J., Muñoz-Pajares, A., Abdelaziz, M., Lorite, J. & Perfectti, F. 2013. Evolution of pollination niches and floral divergence in the generalist plant *Erysimum mediohispanicum*. *Annals of botany*: doi: 10.1093/aob/mct186.

Gómez, J. M., Bosch, J., Perfectti, F., Fernández, J., Abdelaziz, M. & Camacho, J. 2008. Spatial variation in selection on corolla shape in a generalist plant is promoted by the preference patterns of its local pollinators. *Proceedings of the Royal Society B: Biological Sciences* **275**: 2241-2249.

Gómez, J. M. & Perfectti, F. 2010. Evolution of complex traits: the case of *Erysimum* corolla shape. *International Journal of Plant Sciences* **171**: 987-998.

Gómez, J. M., Perfectti, F. & Camacho, J. P. M. 2006. Natural selection on *Erysimum mediohispanicum* flower shape: insights into the evolution of zygomorphy. *The American Naturalist* **168**: 531-545.

Grant, V. 1949. Pollination Systems as Isolating Mechanisms in Angiosperms. *Evolution* **3**: 82-97.

Grant, V. 1981. *Plant Speciation,* 2nd ed. Columbia University Press, New York.

Harmon, L. J., Weir, J. T., Brock, C. D., Glor, R. E. & Challenger, W. 2008. GEIGER: investigating evolutionary radiations. *Bioinformatics* **24**: 129-131.

Heled, J. & Drummond, A. J. 2010. Bayesian Inference of Species Trees from Multilocus Data. *Molecular Biology and Evolution* **27**: 570-580.

Herrera, C. (1996) Floral Traits and Plant Adaptation to Insect Pollinators: A Devil's Advocate Approach. In: *Floral Biology*, (Lloyd, D. & Barrett, S. H., eds.). pp. 65-87. Springer US.

Herrera, C. M. 1993. Selection on floral morphology and environmental determinants of fecundity in a hawk moth-pollinated violet. *Ecological Monographs* **63**: 251-275.

Herrera, C. M., Castellanos, M. C. & Medrano, M. (2006) Geographical context of floral evolution: towards an improved research programme in floral diversification. In: *Ecology and evolution of flowers* (Harder, L. D. & Barrett, S. C. H., eds.). pp. Oxford University press, Oxford.

Hill, E. J. 1909. Pollination in *Linaria* with special reference to cleistogamy. *Botanical Gazette* **47**: 454-466.

Hodges, S. A. 1997. Floral nectar spurs and diversification. *International Journal of Plant Sciences* **158**: S81-S88.

Hodges, S. A. & Arnold, M. L. 1994. Floral and ecological isolation between *Aquilegia formosa* and *Aquilegia pubescens*. *Proceedings of the National Academy of Sciences* **91**: 2493-2496.

Hodges, S. A. & Arnold, M. L. 1995. Spurring plant diversification: are floral nectar spurs a key innovation? *Proceedings of the Royal Society of London. Series B: Biological Sciences* **262**: 343-348.

Johnson, S. & Steiner, K. 1997. Long-tongued fly pollination and evolution of floral spur length in the *Disa draconis* complex (Orchidaceae). *Evolution* **51**: 45-53.

Johnson, S. D. & Steiner, K. E. 2000. Generalization versus specialization in plant pollination systems. *Trends in Ecology & Evolution* **15**: 140-143.

Kampny, C. M. 1995. Pollination and flower diversity in Scrophulariaceae. *The Botanical Review* **61**: 350-366.

Kay, K. M. & Sargent, R. D. 2009. The role of animal pollination in plant speciation: integrating ecology, geography, and genetics. *Annual Review of Ecology, Evolution, and Systematics* **40**: 637-656.

Kephart, S. & Theiss, K. 2004. Pollinator-mediated isolation in sympatric milkweeds (*Asclepias*): do floral morphology and insect behavior influence species boundaries? *New Phytologist* **161**: 265-277.

Knuth, P. 1909. *Handbook of Flower Pollination: Based Upon Hermann Müller's Work 'The*

Fertilisation of Flowers by Insects'. Clarendon Press, Oxford.

Leaché, A. D., Harris, R. B., Rannala, B. & Yang, Z. 2013. The Influence of Gene Flow on Species Tree Estimation: A Simulation Study. *Systematic Biology* **doi: 10.1093/sysbio/syt049**.

Lehrer, M., Horridge, G., Zhang, S. & Gadagkar, R. 1995. Shape vision in bees: innate preference for flower-like patterns. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences* **347**: 123-137.

Maddison, W. P. & Maddison, D. R. (2011) Mesquite: a modular system for evolutionary analysis. Version 2.75 http://mesquiteproject.org. Accessed July 2013. pp.

Magallón, S. & Sanderson, M. J. 2001. Absolute diversification rates in angiosperm clades. *Evolution* **55**: 1762-1780.

Møller, A. P. 1995. Bumblebee preference for symmetrical flowers. *Proceedings of the National Academy of Sciences* **92**: 2288-2292.

Müller, H. 1873. *Die befruchtung der blumen durch insekten und die gegeseitigen anpassungen beider: Ein beitrag zur erkenntniss des ursächlichen zusammenhanges in der organischen natur*. W. Engelmann.

Neal, P. R., Dafni, A. & Giurfa, M. 1998. Floral symmetry and its role in plant-pollinator systems: terminology, distribution, and hypotheses. *Annual Review of Ecology and Systematics* **29**: 345-373.

Nilsson, L. A. 1988. The evolution of flowers with deep corolla tubes. *Nature* **334**: 147-149.

Nunn, C. L. 2011. *The Comparative Approach in Evolutionary Anthropology and Biology*. University of Chicago Press, Chicago.

Oksanen, J., Kindt, R., Legendre, P., O'Hara, B., Stevens, M. H. H., Oksanen, M. J. & Suggests, M. 2007. The vegan package. *Community ecology package. Version*: 1.8-5.

Ollerton, J. 1996. Reconciling Ecological Processes with Phylogenetic Patterns: The Apparent Paradox of Plant--Pollinator Systems. *Journal of Ecology* **84**: 767-769.

Ollerton, J., Alarcón, R., Waser, N. M., Price, M. V., Watts, S., Cranmer, L., Hingston, A., Peter, C. I. & Rotenberry, J. 2009. A global test of the pollination syndrome hypothesis. *Annals of Botany* **103**: 1471-1480.

Pérez-Barrales, R., Arroyo, J. & Scott Armbruster, W. 2007. Differences in pollinator faunas may generate geographic differences in floral morphology and integration in *Narcissus papyraceus* (Amaryllidaceae). *Oikos* **116**: 1904-1918.

Pettersson, M. W. 1991. Pollination by a guild of fluctuating moth populations: option for unspecialization in *Silene vulgaris*. *The Journal of Ecology* **79**: 591-604.

Ree, R. H. & Donoghue, M. J. 1999. Inferring rates of change in flower symmetry in asterid angiosperms. *Systematic Biology* **48**: 633-641.

Robertson, C. 1888. Zygomorphy and Its Causes. I-III. *Botanical Gazette* **13**: 146-151, 203-208, 224-230.

Robertson, J. L. & Wyatt, R. 1990. Evidence for Pollination Ecotypes in the Yellow-Fringed Orchid, *Platanthera ciliaris*. *Evolution* **44**: 121-133.

Rohlf, F. 2010a. tpsDig2 version 2.16. *Stony Brook, Nueva York: Department of Ecology and Evolution, State University*.

Rohlf, F. 2010b. TpsRelw–thin plate spline relative warp analysis. version 1.49. *State University of New York at Stony Brook, New York*.

Rohlf, F. J. 1990. Morphometrics. *Annual Review of Ecology and Systematics* **21**: 299-316.

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M. A. & Huelsenbeck, J. P. 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* **61**: 539-542.

Sáez, L. & Bernal, M. (2009) *Linaria* Mill. In: *Flora Iberica 13*, (Castroviejo, S., Herrero, A., Benedí, C., Rico, E. & Güemes, J., eds.). pp. 232-324. CSIC, Madrid.

Sánchez-Lafuente, A. 2007. Corolla herbivory, pollination success and fruit predation in complex flowers: An experimental study with *Linaria lilacina* (Scrophulariaceae). *Annals of Botany* **99**: 355-364.

Sánchez-Lafuente, A. M., Rodríguez-Gironés, M. A. & Parra, R. 2011. Interaction frequency and per-interaction effects as predictors of total effects in plant–pollinator mutualisms: a case study with the self-incompatible herb *Linaria lilacina*. *Oecologia* **168**: 153-165.

Schemske, D. W. & Bradshaw, H. 1999. Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*).

Proceedings of the National Academy of Sciences **96**: 11910-11915.

Schemske, D. W. & Horvitz, C. C. 1984. Variation among floral visitors in pollination ability: a precondition for mutualism specialization. *Science* **225**: 519-521.

Schnitzler, J., Barraclough, T. G., Boatwright, J. S., Goldblatt, P., Manning, J. C., Powell, M. P., Rebelo, T. & Savolainen, V. 2011. Causes of plant diversification in the Cape biodiversity hotspot of South Africa. *Systematic Biology* **60**: 343-357.

Seehausen, O. 2004. Hybridization and adaptive radiation. *Trends in Ecology and Evolution* **19**: 198-207.

Smith, S. D. 2010. Using phylogenetics to detect pollinator-mediated floral evolution. *New Phytologist* **188**: 354-363.

Stebbins, G. L. 1970. Adaptive Radiation of Reproductive Characteristics in Angiosperms, I: Pollination Mechanisms. *Annual Review of Ecology and Systematics* **1**: 307-326.

Steiner, K. E. & Whitehead, V. B. 1991. Oil Flowers and Oil Bees: Further Evidence for Pollinator Adaptation. *Evolution* **45**: 1493-1501.

Stone, G. N., Nee, S. & Felsenstein, J. 2011. Controlling for non-independence in comparative analysis of patterns across populations within species. *Philosophical Transactions of the Royal Society B: Biological Sciences* **366**: 1410-1424.

Strauss, S. Y., Sahli, H. & Conner, J. K. 2005. Toward a more trait-centered approach to diffuse (co) evolution. *New Phytologist* **165**: 81-90.

Sutton, D. A. 1988. *A revision of the tribe Antirrhineae*. Oxford University Press, London. UK.

Thomson, J. D. & Wilson, P. 2008. Explaining evolutionary shifts between bee and hummingbird pollination: convergence, divergence, and directionality. *International Journal of Plant Sciences* **169**: 23-38.

Valente, L. M., Manning, J. C., Goldblatt, P. & Vargas, P. 2012. Did pollination shifts drive diversification in southern African *Gladiolus*? Evaluating the model of pollinator-driven speciation. *The American Naturalist* **180**: 83-98.

Valente, L. M., Savolainen, V. & Vargas, P. 2010. Unparalleled rates of species diversification in Europe. *Proceedings of the Royal Society B: Biological Sciences* **277**: 1489-1496.

van der Niet, T., Johnson, S. D. & Linder, H. P. 2006. Macroevolutionary data suggest a role for reinforcement in pollination system shifts. *Evolution* **60**: 1596-1601.

van der Niet, T., Pirie, M. D., Shuttleworth, A., Johnson, S. D. & Midgley, J. J. 2013. Do pollinator distributions underlie the evolution of pollination ecotypes in the Cape shrub Erica plukenetii? *Annals of Botany*: mct193.

Vargas, P., Ornosa, C., Ortiz-Sánchez, F. J. & Arroyo, J. 2010. Is the occluded corolla of *Antirrhinum* bee-specialized? *Journal of Natural History* **44**: 1427-1443.

Viano, J. 1969. Note sur le genre *Linaria* en Méditerranée occidentale. Le groupe *Linaria bipartita* (Vent.) Willd. *Naturalia monspeliensia, série Botanique* **20**: 219-240.

Waser, N. M., Chittka, L., Price, M. V., Williams, N. M. & Ollerton, J. 1996. Generalization in Pollination Systems, and Why it Matters. *Ecology* **77**: 1043-1060.

Whittall, J. B. & Hodges, S. A. 2007. Pollinator shifts drive increasingly long nectar spurs in columbine flowers. *Nature* **447**: 706-709.

Wilson, P. & Thomson, J. D. (1996) How Flowers Diverge? In: *Floral Biology: Studies on Floral Evolution in Animal-pollinated Plants*, (Lloyd, D. G. & Barrett, S. C. H., eds.). pp. 88-111. Springer.

Yoder, J., Smith, C., Rowley, D., Flatz, R., Godsoe, W., Drummond, C. & Pellmyr, O. 2013. Effects of gene flow on phenotype matching between two varieties of Joshua tree (*Yucca brevifolia*; Agavaceae) and their pollinators. *Journal of evolutionary biology* **26**.

Zelditch, M. L., Swiderski, D. L. & Sheets, H. D. 2012. *Geometric morphometrics for biologists: a primer*. Academic Press.

Supporting Information from Blanco-Pastor *et al.,* "Bee morphotypes explain floral variation in a radiation of *Linaria* species"

Journal of Evolutionary Biology, submitted

Figure S1

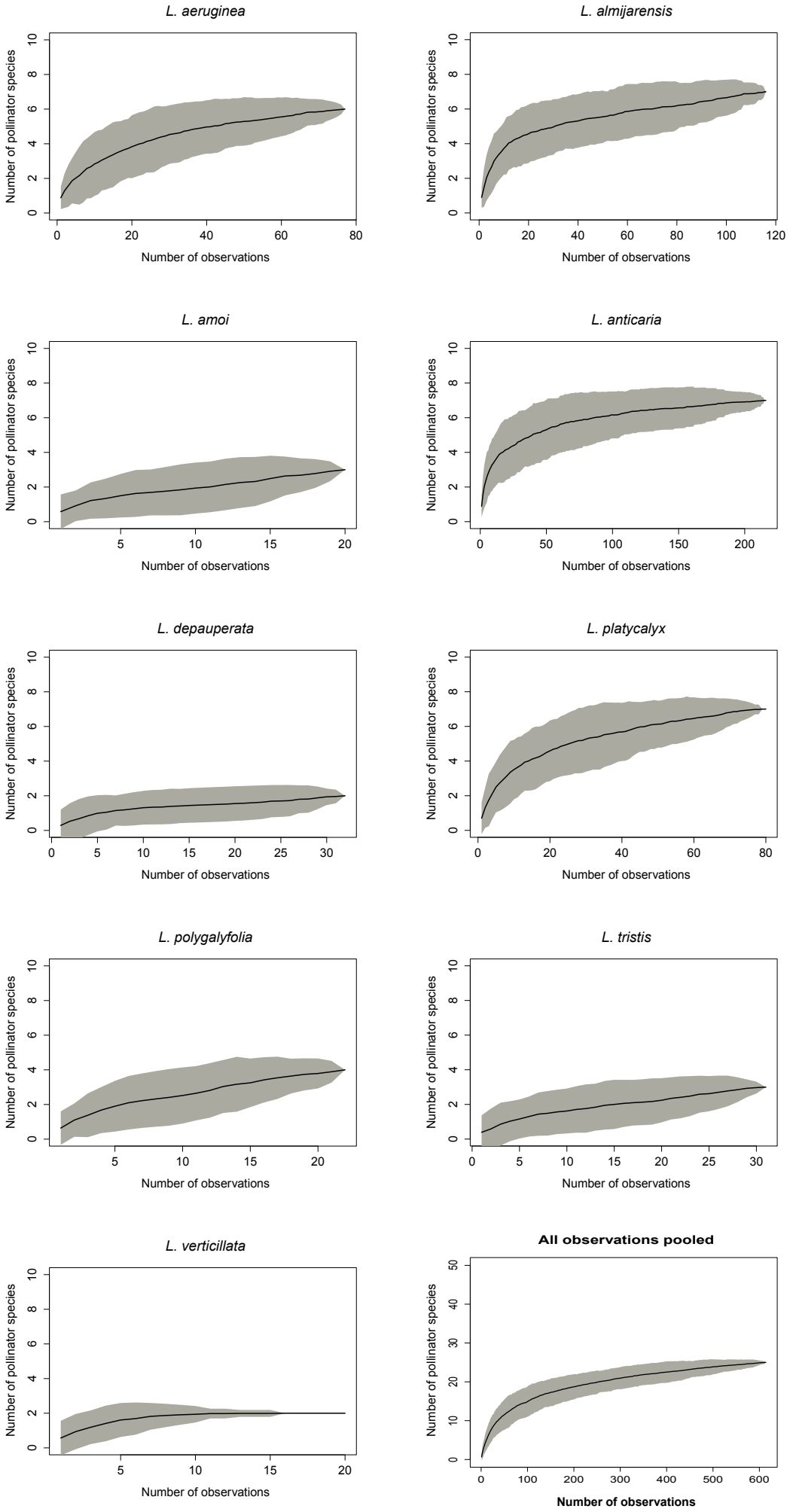
L. aeruginea
L. almijarensis
L. amoi
L. anticaria
L. depauperata
L. platycalyx
L. polygalyfolia
L. tristis
L. verticillata
All observations pooled
Number of pollinator species
Number of observations

Figure S2

RW	DFA	
	1	2
RW12	,417*	,129
RW1	,245*	,063
RW10	,170*	,050
RW9	,168*	,086
RW4	-,168*	-,009
RW13	,140*	,054
RW16	-,123*	,057
RW27	-,087*	-,015
RW14	,080*	,037
RW30	-,069*	-,049
RW22	-,063*	-,010
RW6	-,057*	-,050
RW29	,051*	-,035
RW28	-,050*	,007
RW24	-,039*	-,004
RW20	,016*	,015
RW2	,160	-,535*
RW11	-,026	,319*
RW7	-,027	-,227*
RW3	-,144	,189*
RW15	-,059	,126*
RW25	-,029	-,125*
RW8	,057	,088*
RW23	,003	,063*
RW17	,026	,059*
RW5	-,027	,053*
RW18	-,024	-,052*
RW21	,021	-,037*
RW26	-,013	-,026*
RW19	,019	-,020*

Pooled within-groups correlations between discriminating variables and standardized canonical discriminant functions

Variables ordered by absolute size of correlation within function.

*. Largest absolute correlation between each variable and any discriminant function

Supplementary Table 1. Morphometric variables of *Linaria* specimens

Linaria species	Year of sampling	Individual No.	Photo No.	Pollinator group	Flower size (mm)	Relative Warps																											
						RW1	RW2	RW3	RW4	RW5	RW6	RW7	RW8	RW9	RW10	RW11	RW12	RW13	RW14	RW15	RW16	RW17	RW18	RW19	RW20	RW21	RW22	RW23	RW24	RW25	RW26	RW27	RW28	RW29	RW30
L. aeruginea	2010	1	IMG2529	Megachilidae	17.66	-0.0113	-0.0550	-0.0796	-0.0220	-0.0004	-0.0086	0.0135	0.0176	-0.0106	-0.0130	0.0085	-0.0190	-0.0111	-0.0088	-0.0033	0.0060	-0.0023	0.0016	0.0051	0.0048	0.0023	-0.0004	0.0006	-0.0007	-0.0011	-0.0004	0.0000	0.0002	-0.0001	-0.0004
L. aeruginea	2010	2	IMG2532	Megachilidae	16.50	0.0022	-0.0489	-0.0843	0.0079	0.0053	0.0040	0.0130	0.0084	0.0016	-0.0078	0.0053	-0.0082	-0.0037	-0.0008	0.0001	0.0014	-0.0007	-0.0010	0.0014	0.0024	0.0010	0.0002	0.0007	0.0001	-0.0009	0.0004	-0.0003	0.0002	0.0001	-0.0001
L. aeruginea	2010	3	IMG2536	Megachilidae	16.43	-0.0293	-0.0702	-0.0977	-0.0086	-0.0119	-0.0145	0.0179	0.0045	-0.0147	-0.0146	0.0009	-0.0131	-0.0142	-0.0055	-0.0067	-0.0007	0.0055	-0.0013	-0.0045	0.0021	-0.0009	-0.0023	0.0012	0.0003	-0.0022	0.0002	-0.0005	0.0014	0.0001	0.0005
L. aeruginea	2010	4	IMG2539	Megachilidae	15.79	0.1265	0.0036	-0.0511	0.0674	0.0060	-0.0155	-0.0337	-0.0028	0.0032	-0.0162	-0.0083	-0.0132	-0.0079	0.0051	0.0042	0.0031	0.0035	0.0017	0.0007	0.0013	0.0020	-0.0009	0.0009	0.0027	0.0002	-0.0019	0.0006	-0.0005	-0.0003	0.0005
L. aeruginea	2010	5	IMG2542	Megachilidae	14.91	0.0915	-0.0685	-0.0701	0.0453	0.0261	-0.0045	0.0026	0.0130	-0.0136	0.0048	0.0001	-0.0158	-0.0022	0.0076	-0.0068	-0.0001	0.0035	-0.0011	-0.0029	-0.0046	-0.0046	0.0003	0.0009	0.0009	-0.0015	-0.0001	-0.0004	-0.0006	-0.0006	0.0010
L. aeruginea	2010	6	IMG2548	Megachilidae	15.29	0.1249	-0.0257	-0.0534	0.0203	0.0049	0.0302	0.0004	0.0074	-0.0054	-0.0070	-0.0001	-0.0071	-0.0080	0.0066	0.0063	-0.0014	0.0010	0.0041	0.0010	-0.0018	0.0007	0.0011	-0.0003	-0.0003	-0.0001	-0.0009	0.0002	0.0004	0.0004	0.0001
L. aeruginea	2010	7	IMG2564	Megachilidae	13.13	0.0403	-0.0455	-0.0718	-0.0085	0.0368	-0.0075	-0.0030	0.0152	-0.0162	-0.0117	0.0096	-0.0090	-0.0073	-0.0088	-0.0041	0.0102	-0.0009	0.0025	-0.0018	0.0029	0.0010	-0.0006	0.0012	0.0008	-0.0017	0.0007	0.0004	0.0002	0.0000	0.0001
L. aeruginea	2010	8	IMG2569	Megachilidae	14.14	0.1201	-0.0674	-0.0614	-0.0115	-0.0153	-0.0164	0.0117	-0.0006	-0.0197	-0.0065	0.0070	-0.0127	-0.0051	-0.0028	0.0000	-0.0026	-0.0022	0.0014	0.0010	-0.0009	0.0013	0.0011	-0.0007	-0.0009	-0.0003	0.0003	-0.0002	0.0001	-0.0003	-0.0002
L. aeruginea	2010	9	IMG2572	Megachilidae	13.75	0.1319	-0.0563	-0.0533	0.0059	0.0038	-0.0118	0.0206	0.0120	-0.0180	0.0033	-0.0042	-0.0153	-0.0041	0.0007	0.0019	0.0045	0.0047	0.0011	0.0044	-0.0018	-0.0002	-0.0001	0.0000	-0.0003	-0.0011	-0.0007	-0.0002	-0.0002	-0.0008	0.0000
L. aeruginea	2010	10	IMG2575	Megachilidae	15.26	0.0452	-0.0959	-0.0843	-0.0252	-0.0115	-0.0216	0.0258	0.0060	-0.0203	-0.0082	-0.0009	-0.0127	-0.0036	0.0015	-0.0063	0.0009	-0.0023	0.0024	0.0003	0.0025	-0.0007	0.0009	0.0013	0.0000	0.0002	0.0003	-0.0001	-0.0001	-0.0002	-0.0001
L. aeruginea	2010	11	IMG2581	Megachilidae	15.81	0.0901	-0.0410	-0.0781	0.0011	0.0026	-0.0061	-0.0010	0.0073	-0.0207	-0.0108	0.0224	-0.0179	-0.0027	0.0095	0.0037	0.0014	-0.0053	0.0029	-0.0042	-0.0047	-0.0012	0.0003	-0.0018	0.0007	0.0008	-0.0001	0.0008	-0.0002	-0.0002	0.0007
L. aeruginea	2010	12	IMG2584	Megachilidae	16.02	0.0908	-0.0567	-0.0583	0.0223	0.0083	-0.0159	0.0201	0.0096	-0.0084	0.0005	-0.0021	-0.0097	-0.0060	0.0062	0.0124	-0.0015	-0.0019	-0.0018	-0.0006	-0.0030	-0.0007	-0.0002	-0.0005	0.0000	0.0002	0.0000	-0.0003	-0.0001	-0.0003	0.0005
L. aeruginea	2010	13	IMG2590	Megachilidae	16.30	0.1321	0.0000	-0.0792	0.0197	-0.0047	0.0164	0.0138	0.0078	-0.0012	-0.0219	0.0139	-0.0062	-0.0083	0.0038	-0.0011	-0.0006	0.0005	0.0057	0.0019	-0.0006	0.0013	0.0012	-0.0001	0.0002	-0.0006	0.0004	-0.0001	0.0000	0.0004	-0.0003
L. aeruginea	2010	14	IMG2600	Megachilidae	14.13	0.0418	-0.0339	-0.0546	0.0398	0.0149	-0.0066	0.0054	0.0139	-0.0165	0.0102	0.0000	-0.0166	-0.0091	0.0068	-0.0058	0.0037	-0.0019	0.0004	0.0028	0.0000	-0.0019	0.0008	0.0012	-0.0005	-0.0001	-0.0001	-0.0006	0.0000	-0.0005	-0.0003
L. aeruginea	2010	15	IMG2609	Megachilidae	15.01	0.1114	-0.0689	-0.0863	0.0173	0.0086	0.0115	0.0249	0.0269	0.0010	0.0093	0.0012	-0.0074	-0.0065	0.0097	0.0082	0.0022	-0.0079	0.0002	-0.0025	-0.0041	-0.0024	0.0014	-0.0030	-0.0012	0.0014	0.0002	0.0006	0.0001	0.0007	-0.0001
L. aeruginea	2010	16	DPP_025	Megachilidae	18.97	-0.0837	-0.0903	-0.0519	0.0044	-0.0221	0.0395	-0.0296	0.0095	-0.0033	0.0003	0.0081	0.0183	-0.0209	-0.0046	0.0014	-0.0065	0.0017	-0.0008	0.0004	0.0027	-0.0018	0.0014	0.0004	0.0000	0.0002	-0.0001	0.0013	0.0006	-0.0008	0.0007
L. aeruginea	2010	17	DPP_032	Megachilidae	21.99	-0.0460	0.0331	-0.0693	0.0210	0.0147	0.0133	-0.0125	-0.0026	-0.0031	-0.0058	0.0063	0.0047	0.0021	-0.0075	0.0034	-0.0016	0.0073	0.0008	-0.0016	0.0008	-0.0009	0.0004	-0.0001	0.0016	-0.0006	-0.0005	-0.0005	0.0008	-0.0001	0.0000
L. aeruginea	2010	18	DPP_044	Megachilidae	21.36	-0.1526	-0.0145	-0.0445	0.0126	-0.0241	0.0254	0.0044	-0.0248	-0.0182	0.0059	0.0107	0.0102	0.0044	-0.0026	0.0002	-0.0032	0.0034	-0.0008	-0.0011	-0.0010	-0.0020	-0.0022	-0.0009	0.0042	-0.0007	-0.0014	-0.0007	0.0008	0.0002	-0.0002
L. aeruginea	2010	19	DPP_047	Megachilidae	20.69	-0.0812	-0.0068	-0.0620	-0.0036	-0.0225	0.0097	-0.0035	-0.0231	-0.0162	0.0035	0.0086	0.0002	-0.0001	-0.0011	0.0029	-0.0066	0.0054	-0.0060	0.0001	0.0020	-0.0015	-0.0014	-0.0010	0.0029	-0.0010	-0.0012	-0.0013	0.0011	0.0009	0.0000
L. aeruginea	2010	20	DPP_053	Megachilidae	20.62	-0.0310	-0.0122	-0.0235	0.0166	-0.0206	0.0140	-0.0103	0.0114	0.0019	0.0226	-0.0007	-0.0039	-0.0066	-0.0064	-0.0104	-0.0029	0.0039	0.0051	0.0017	-0.0005	0.0014	0.0012	0.0014	-0.0004	-0.0009	0.0010	-0.0003	-0.0005	-0.0002	-0.0001
L. aeruginea	2010	21	DPP_057	Megachilidae	19.22	-0.0315	0.0235	-0.0771	0.0464	-0.0325	-0.0051	-0.0014	0.0041	-0.0151	0.0061	0.0048	-0.0048	-0.0118	-0.0001	0.0008	0.0067	0.0059	0.0024	-0.0034	-0.0010	0.0039	-0.0002	0.0004	-0.0010	-0.0006	0.0009	-0.0008	0.0003	-0.0007	0.0003
L. aeruginea	2010	22	DPP_063	Megachilidae	17.24	0.0461	0.0057	-0.0058	-0.0502	-0.0045	0.0170	0.0194	-0.0035	-0.0334	0.0067	0.0042	-0.0031	0.0046	-0.0099	0.0015	-0.0089	0.0034	-0.0015	-0.0012	-0.0002	0.0020	0.0003	-0.0002	-0.0003	0.0009	0.0005	0.0006	-0.0007	-0.0002	0.0000
L. aeruginea	2010	23	DPP_069	Megachilidae	16.45	0.0213	-0.0227	0.0030	-0.0091	-0.0115	0.0425	0.0143	0.0126	-0.0038	0.0102	0.0110	-0.0050	-0.0095	0.0001	0.0065	0.0013	-0.0023	0.0023	-0.0021	-0.0037	0.0039	0.0009	-0.0028	0.0001	0.0002	0.0002	0.0001	0.0001	0.0001	-0.0001
L. aeruginea	2010	24	DPP_072	Megachilidae	17.88	0.0584	-0.0246	-0.0389	0.0298	-0.0295	-0.0102	0.0061	-0.0097	0.0018	-0.0019	0.0092	-0.0052	-0.0023	0.0017	-0.0073	-0.0093	-0.0022	-0.0065	-0.0038	0.0039	-0.0018	0.0016	-0.0005	-0.0003	-0.0002	0.0011	0.0004	-0.0002	0.0003	-0.0002
L. aeruginea	2010	25	DPP_078	Megachilidae	21.85	-0.0221	0.0358	-0.0216	-0.0271	0.0191	-0.0150	0.0060	0.0088	-0.0068	0.0006	0.0063	0.0009	0.0025	0.0022	0.0006	-0.0026	0.0042	0.0002	-0.0016	0.0001	-0.0009	-0.0008	0.0003	0.0001	-0.0001	-0.0002	0.0000	0.0002	0.0000	0.0000
L. aeruginea	2010	26	IMG2545	Megachilidae	15.59	0.1332	-0.0451	-0.0789	0.0018	-0.0042	-0.0155	0.0009	0.0304	-0.0038	-0.0141	0.0061	-0.0091	-0.0081	0.0055	-0.0015	-0.0127	0.0003	0.0014	-0.0035	-0.0021	0.0008	-0.0023	0.0014	0.0000	0.0017	-0.0003	0.0002	-0.0003	0.0003	-0.0004
L. almijarensis	2010	1	IMG1572b	Megachilidae	18.93	-0.0758	0.0128	0.0064	-0.0584	-0.0208	0.0678	-0.0093	-0.0144	-0.0146	-0.0027	-0.0004	-0.0169	-0.0029	0.0180	-0.0081	0.0092	-0.0021	-0.0007	0.0003	0.0014	0.0014	0.0002	0.0011	0.0030	0.0011	0.0002	0.0000	0.0005	-0.0001	0.0000
L. almijarensis	2010	2	IMG1575b	Megachilidae	18.46	-0.0139	-0.0129	-0.0313	-0.0331	-0.0158	0.0096	0.0186	-0.0002	0.0054	-0.0153	-0.0077	-0.0128	-0.0068	0.0000	0.0057	0.0036	0.0072	0.0000	0.0037	-0.0002	0.0009	0.0003	0.0009	-0.0004	-0.0006	-0.0002	-0.0001	0.0000	0.0001	0.0000
L. almijarensis	2010	3	IMG1578b	Megachilidae	18.94	0.0551	-0.0280	0.0118	0.0129	-0.0149	0.0091	0.0341	-0.0153	0.0147	-0.0102	-0.0064	0.0039	-0.0042	0.0028	0.0030	0.0028	-0.0034	0.0000	0.0008	0.0024	-0.0002	0.0012	0.0000	0.0007	-0.0013	-0.0002	-0.0004	0.0004	0.0000	0.0001
L. almijarensis	2010	4	IMG1583	Megachilidae	22.49	-0.0696	-0.0079	-0.0250	-0.0509	0.0007	0.0330	0.0031	0.0085	0.0117	-0.0070	0.0010	-0.0061	-0.0012	-0.0005	0.0016	0.0036	-0.0010	-0.0018	0.0016	0.0006	0.0010	0.0000	0.0009	0.0004	-0.0003	-0.0001	-0.0004	-0.0002	0.0001	0.0003
L. almijarensis	2010	5	IMG1589	Megachilidae	21.05	-0.0560	0.0102	-0.0255	-0.0377	0.0135	0.0373	-0.0076	0.0037	-0.0084	-0.0075	-0.0046	-0.0048	-0.0061	0.0088	0.0034	-0.0031	-0.0088	-0.0016	-0.0025	0.0017	0.0009	0.0000	0.0004	0.0000	-0.0007	-0.0005	0.0002	-0.0001	-0.0002	-0.0002
L. almijarensis	2010	6	IMG1595	Megachilidae	19.13	0.0008	-0.0744	0.0409	0.0253	-0.0036	-0.0066	-0.0073	-0.0022	-0.0168	0.0247	0.0040	-0.0090	-0.0003	-0.0024	-0.0045	-0.0046	0.0009	-0.0006	-0.0017	-0.0022	-0.0006	0.0011	0.0012	0.0004	-0.0010	0.0001	-0.0003	-0.0001	-0.0001	0.0000
L. almijarensis	2010	7	IMG1601	Megachilidae	19.94	0.0113	-0.0363	0.0151	0.0151	-0.0179	0.0099	0.0055	-0.0100	-0.0138	0.0120	-0.0050	-0.0112	0.0002	0.0035	0.0040	-0.0051	0.0031	-0.0019	0.0028	0.0003	0.0015	0.0016	0.0005	0.0001	-0.0007	0.0002	0.0001	-0.0002	-0.0002	0.0000
L. almijarensis	2010	8	IMG1608	Megachilidae	19.29	-0.0679	-0.0876	0.0685	0.0194	-0.0420	0.0155	0.0155	0.0009	-0.0066	-0.0065	0.0164	-0.0056	0.0006	0.0038	0.0024	-0.0057	-0.0021	-0.0016	-0.0061	0.0014	0.0021	0.0014	0.0000	0.0003	0.0001	-0.0008	0.0003	0.0002	0.0000	-0.0003
L. almijarensis	2010	9	IMG1611	Megachilidae	19.96	-0.0431	-0.0292	-0.0367	-0.0291	0.0170	0.0335	-0.0058	0.0056	0.0063	-0.0152	-0.0034	-0.0073	-0.0052	-0.0022	0.0048	0.0020	0.0041	-0.0019	-0.0041	0.0000	0.0005	0.0005	0.0012	0.0007	-0.0008	0.0000	-0.0002	0.0000	0.0002	-0.0003
L. almijarensis	2010	10	IMG1626	Megachilidae	22.09	-0.0083	-0.0184	0.0012	-0.0241	-0.0074	-0.0014	-0.0291	0.0078	0.0021	0.0119	-0.0032	-0.0085	-0.0116	0.0020	-0.0027	0.0006	0.0013	-0.0039	0.0021	0.0022	0.0008	-0.0002	-0.0003	-0.0001	-0.0001	-0.0002	-0.0003	0.0000	-0.0002	-0.0003
L. almijarensis	2010	11	IMG1638	Megachilidae	21.16	-0.0234	-0.0389	0.0062	0.0164	-0.0121	0.0335	0.0181	-0.0066	0.0051	0.0138	0.0111	-0.0081	0.0094	0.0064	0.0023	-0.0012	0.0006	-0.0034	-0.0053	-0.0003	0.0007	0.0010	0.0000	0.0010	-0.0005	-0.0006	0.0004	0.0000	0.0000	-0.0001
L. almijarensis	2010	12	IMG1641	Megachilidae	22.58	-0.0339	0.0011	-0.0117	-0.0040	0.0062	0.0263	0.0136	-0.0074	0.0098	0.0029	0.0019	-0.0074	-0.0044	0.0006	0.0043	0.0035	-0.0048	0.0026	0.0035	0.0018	0.0008	0.0005	0.0012	0.0000	-0.0011	0.0002	-0.0004	-0.0004	0.0003	0.0002
L. almijarensis	2010	13	IMG1644	Megachilidae	21.70	-0.0536	0.0169	0.0002	-0.0114	0.0031	0.0117	0.0150	-0.0107	-0.0040	0.0111	0.0066	-0.0131	-0.0033	0.0034	-0.0026	0.0033	-0.0025	-0.0026	0.0007	0.0024	0.0003	-0.0005	0.0006	0.0007	-0.0006	-0.0010	0.0001	-0.0001	0.0003	0.0000
L. almijarensis	2012	14	IMG1061	Megachilidae	20.30	-0.0693	0.0017	0.0607	-0.0084	-0.0243	0.0035	-0.0171	0.0245	0.0158	-0.0162	0.0139	-0.0043	-0.0015	0.0080	-0.0023	-0.0042	0.0048	-0.0026	0.0029	-0.0005	-0.0005	0.0006	-0.0002	-0.0012	0.0000	0.0003	-0.0006	-0.0002	-0.0002	0.0006
L. almijarensis	2012	15	IMG1064	Megachilidae	20.97	0.0238	0.0051	0.0357	0.0120	0.0174	0.0079	-0.0090	0.0205	0.0022	-0.0183	0.0000	0.0012	0.0040	-0.0087	-0.0017	0.0001	-0.0038	-0.0009	0.0062	0.0046	-0.0002	-0.0007	0.0004	-0.0004	-0.0009	0.0005	-0.0001	0.0005	-0.0005	0.0000
L. almijarensis	2012	16	IMG1069	Megachilidae	19.73	0.0411	0.0338	0.0412	0.0138	-0.0331	-0.0067	-0.0075	0.0167	-0.0042	-0.0110	-0.0067	-0.0112	-0.0112	-0.0011	-0.0007	0.0094	0.0017	0.0057	0.0009	-0.0021	0.0009	-0.0002	-0.0020	0.0018	0.0014	-0.0006	0.0004	0.0001	0.0003	-0.0001
L. almijarensis	2012	17	IMG1072	Megachilidae	20.71	-0.0804	0.0179	0.0167	0.0002	-0.0260	0.0066	0.0090	0.0261	0.0099	-0.0095	-0.0066	-0.0039	0.0046	-0.0042	-0.0003	0.0019	0.0011	0.0003	-0.0039	-0.0027	-0.0025	0.0014	0.0004	-0.0007	0.0002	0.0001	0.0002	0.0005	-0.0003	-0.0002
L. almijarensis	2012	18	IMG1075	Megachilidae	19.58	0.0112	-0.0106	0.0589	0.0564	-0.0310	-0.0137	-0.0089	0.0023	0.0167	0.0047	0.0095	-0.0038	-0.0099	-0.0118	-0.0021	-0.0029	-0.0107	0.0054	-0.0052	0.0012	0.0004	0.0034	-0.0002	-0.0007	0.0007	0.0004	-0.0005	-0.0004	0.0001	0.0003
L. almijarensis	2012	19	IMG1078	Megachilidae	18.48	-0.1000	0.0114	-0.0037	0.0078	-0.0166	0.0032	-0.0102	0.0078	-0.0004	-0.0143	0.0093	-0.0088	-0.0006	-0.0038	0.0044	-0.0027	-0.0008	0.0039	0.0008	-0.0016	0.0004	0.0010	0.0009	-0.0002	0.0002	-0.0006	-0.0002	-0.0003	0.0001	0.0000
L. almijarensis	2012	20	IMG1081	Megachilidae	22.18	-0.1038	-0.0183	0.0018	0.0053	-0.0367	0.0309	-0.0091	0.0166	-0.0203	-0.0005	-0.0008	-0.0104	0.0127	-0.0092	0.0072	0.0001	-0.0031	0.0024	0.0016	-0.0018	0.0021	-0.0011	-0.0022	-0.0004	0.0003	-0.0008	-0.0002	0.0007	0.0005	0.0001
L. almijarensis	2012	21	IMG1084	Megachilidae	20.99	0.0286	0.0255	0.0511	-0.0034	-0.0265	-0.0114	-0.0064	0.0074	-0.0023	-0.0161	0.0099	-0.0082	-0.0119	-0.0003	-0.0054	0.0087	-0.0009	0.0006	0.0030	0.0000	-0.0023	0.0008	-0.0020	0.0018	0.0000	-0.0004	0.0001	0.0004	-0.0001	-0.0001

L. almijarensis	2012	22	IMG1087	Megachilidae	21.00	-0.0872	-0.0138	0.0135	0.0212	-0.0339	-0.0040	-0.0119	0.0117	-0.0176	-0.0002	0.0028	0.0039	-0.0001	-0.0058	0.0012	-0.0065	-0.0010	0.0028	0.0012	-0.0004	0.0009	0.0002	-0.0012	-0.0009	0.0000	0.0000	-0.0003	-0.0002	0.0001	0.0000
L. almijarensis	2012	23	IMG1090	Megachilidae	20.03	-0.0308	-0.0024	0.0215	0.0491	-0.0365	-0.0169	0.0076	0.0054	0.0131	-0.0054	-0.0042	-0.0098	-0.0045	-0.0010	-0.0013	0.0013	-0.0053	0.0016	0.0040	0.0038	0.0018	0.0005	0.0014	-0.0008	0.0004	0.0005	-0.0002	-0.0002	-0.0002	0.0001
L. almijarensis	2012	24	IMG1093	Megachilidae	21.07	-0.0818	0.0255	0.0242	-0.0119	-0.0183	-0.0055	-0.0098	0.0135	0.0114	-0.0070	0.0068	-0.0051	0.0159	0.0040	-0.0003	-0.0028	-0.0085	0.0018	-0.0009	0.0014	-0.0011	-0.0014	0.0002	0.0004	-0.0017	0.0002	0.0003	0.0005	-0.0008	0.0004
L. almijarensis	2012	25	IMG1096	Megachilidae	20.91	-0.1352	0.0167	0.0213	0.0223	-0.0399	0.0030	0.0025	0.0096	0.0120	0.0046	0.0056	-0.0085	0.0026	-0.0126	-0.0004	0.0021	-0.0055	-0.0039	0.0000	-0.0070	-0.0051	-0.0008	0.0015	0.0006	0.0016	-0.0004	-0.0011	0.0000	-0.0008	0.0000
L. almijarensis	2012	26	IMG1155	Megachilidae	18.60	0.0277	-0.0326	0.0573	0.0204	-0.0362	0.0070	-0.0103	0.0208	0.0055	-0.0076	-0.0082	-0.0112	0.0160	-0.0020	0.0027	-0.0012	-0.0004	0.0049	-0.0015	0.0046	0.0031	-0.0015	0.0012	-0.0011	0.0003	-0.0019	0.0003	0.0017	0.0003	0.0006
L. almijarensis	2012	27	IMG1159	Megachilidae	21.70	-0.0526	-0.0321	-0.0063	0.0266	-0.0228	0.0015	-0.0045	0.0098	0.0057	-0.0066	-0.0022	-0.0038	-0.0017	0.0045	0.0091	0.0021	-0.0108	-0.0030	-0.0019	0.0007	0.0020	-0.0004	-0.0004	-0.0006	-0.0003	-0.0003	-0.0001	-0.0007	0.0001	-0.0002
L. almijarensis	2012	28	IMG1163	Megachilidae	20.66	-0.0313	0.0080	-0.0004	0.0512	-0.0316	-0.0316	0.0153	-0.0148	-0.0062	0.0030	-0.0013	-0.0020	0.0095	-0.0154	-0.0011	0.0020	-0.0108	-0.0003	-0.0041	0.0003	-0.0003	-0.0017	0.0005	-0.0008	-0.0013	0.0000	-0.0004	-0.0006	0.0001	0.0001
L. almijarensis	2012	29	IMG1166	Megachilidae	19.61	-0.1032	0.0008	0.0060	0.0144	-0.0129	-0.0140	0.0271	0.0270	0.0280	-0.0064	0.0005	-0.0023	-0.0070	-0.0171	0.0040	-0.0063	0.0014	0.0015	0.0043	-0.0096	-0.0080	0.0016	0.0018	0.0001	0.0024	-0.0007	-0.0009	-0.0002	-0.0004	0.0003
L. almijarensis	2012	30	IMG1170	Megachilidae	21.94	-0.0762	0.0212	-0.0132	0.0067	-0.0136	-0.0192	0.0026	0.0213	0.0249	-0.0251	0.0039	-0.0034	-0.0004	-0.0021	0.0015	0.0037	0.0028	-0.0013	0.0015	-0.0049	-0.0029	0.0017	0.0017	-0.0008	0.0016	-0.0001	-0.0010	-0.0002	-0.0002	-0.0002
L. almijarensis	2012	31	IMG1173	Megachilidae	18.59	-0.0630	0.0123	0.0262	-0.0033	0.0070	0.0141	0.0224	0.0087	-0.0207	-0.0259	-0.0177	-0.0075	-0.0034	-0.0110	0.0003	-0.0021	-0.0043	0.0031	-0.0033	0.0000	-0.0054	0.0020	0.0013	0.0000	-0.0012	0.0015	0.0015	-0.0004	0.0011	-0.0003
L. almijarensis	2012	32	IMG1176	Megachilidae	19.94	-0.1314	0.0079	0.0075	0.0040	-0.0265	-0.0022	0.0285	0.0049	-0.0034	-0.0079	0.0011	-0.0052	0.0018	0.0013	0.0012	-0.0009	0.0010	-0.0020	-0.0002	-0.0005	-0.0004	0.0002	0.0011	-0.0012	-0.0005	0.0002	-0.0009	0.0000	0.0006	0.0000
L. almijarensis	2012	33	IMG1179	Megachilidae	20.61	-0.0472	0.0249	0.0524	0.0071	-0.0152	0.0174	0.0089	0.0166	0.0137	-0.0113	0.0046	0.0000	0.0019	0.0093	0.0096	0.0039	0.0016	0.0038	-0.0019	-0.0028	0.0010	0.0016	0.0002	-0.0010	-0.0003	-0.0002	-0.0003	0.0005	0.0001	-0.0006
L. almijarensis	2012	34	IMG1182	Megachilidae	20.98	-0.1008	-0.0361	0.0354	0.0438	-0.0425	0.0048	-0.0044	0.0163	-0.0017	0.0016	-0.0004	0.0004	0.0051	-0.0169	-0.0032	0.0020	0.0022	0.0021	0.0003	-0.0039	-0.0032	0.0000	-0.0002	0.0003	0.0004	0.0003	0.0001	0.0000	0.0000	-0.0002
L. almijarensis	2012	35	IMG1185	Megachilidae	21.21	-0.0567	-0.0098	0.0294	0.0153	-0.0425	-0.0089	-0.0029	0.0132	-0.0184	-0.0090	-0.0105	-0.0174	0.0036	-0.0106	0.0088	0.0045	0.0068	0.0014	0.0014	-0.0051	-0.0003	0.0007	-0.0007	0.0004	0.0012	0.0009	0.0014	-0.0001	0.0013	-0.0001
L. amoi	2010	1	IMG1967	Megachilidae	24.62	-0.0917	-0.0529	-0.0476	0.0469	-0.0712	0.0538	0.0027	-0.0601	-0.0340	-0.0081	-0.0001	0.0342	-0.0056	0.0004	0.0023	0.0121	0.0021	0.0058	-0.0022	-0.0044	0.0006	-0.0026	0.0014	-0.0038	0.0001	-0.0005	0.0013	-0.0005	-0.0012	-0.0004
L. amoi	2010	2	IMG1970	Megachilidae	22.91	-0.0686	0.0092	-0.0458	0.0331	-0.0283	0.0301	-0.0069	-0.0150	-0.0150	0.0038	0.0035	0.0064	0.0020	-0.0069	0.0040	0.0012	0.0034	0.0018	0.0039	-0.0011	0.0025	0.0016	0.0006	0.0008	-0.0001	0.0007	-0.0004	-0.0001	-0.0001	-0.0002
L. amoi	2010	3	IMG1978	Megachilidae	23.66	0.0213	0.0243	-0.0435	0.0253	-0.0253	0.0182	-0.0080	-0.0087	-0.0091	0.0067	-0.0014	-0.0028	0.0077	-0.0008	0.0057	-0.0009	-0.0003	-0.0002	0.0046	-0.0009	0.0015	0.0008	-0.0009	0.0009	-0.0006	0.0014	0.0001	0.0002	0.0003	-0.0002
L. amoi	2010	4	IMG1982	Megachilidae	23.21	-0.1576	-0.0229	-0.0381	0.0462	0.0138	0.0313	0.0124	-0.0008	0.0073	0.0356	0.0247	0.0131	-0.0076	0.0077	0.0054	-0.0018	0.0108	0.0070	0.0019	0.0117	-0.0008	0.0003	0.0021	0.0011	0.0014	0.0026	0.0022	-0.0003	-0.0008	0.0004
L. amoi	2010	5	IMG1991	Megachilidae	23.29	-0.1048	0.0551	-0.0499	0.0524	0.0182	0.0410	-0.0119	-0.0011	-0.0018	-0.0027	0.0026	0.0062	0.0044	-0.0175	-0.0048	0.0066	0.0038	0.0047	0.0034	-0.0034	0.0013	0.0014	0.0016	0.0009	0.0013	0.0005	0.0000	-0.0001	-0.0002	-0.0002
L. amoi	2010	6	IMG1983	Megachilidae	22.19	-0.1390	-0.0177	-0.0174	0.0251	-0.0182	0.0165	0.0006	0.0050	-0.0086	0.0421	-0.0022	0.0022	-0.0029	0.0090	-0.0069	0.0045	-0.0057	-0.0088	0.0000	0.0073	-0.0007	-0.0003	-0.0003	-0.0014	0.0017	-0.0005	0.0009	-0.0009	0.0009	-0.0003
L. amoi	2012	7	IMG1316	Megachilidae	21.95	-0.0617	0.0388	0.0144	0.0497	-0.0324	0.0218	0.0099	0.0044	-0.0154	-0.0043	0.0104	0.0035	0.0076	-0.0047	0.0003	-0.0083	-0.0047	-0.0090	-0.0033	0.0012	-0.0016	-0.0011	-0.0020	0.0002	0.0003	-0.0002	0.0000	0.0008	-0.0002	0.0000
L. amoi	2012	8	IMG1319b	Megachilidae	21.31	0.0167	0.0524	0.0336	0.0451	-0.0074	-0.0160	-0.0008	0.0061	-0.0185	-0.0086	-0.0021	-0.0047	0.0057	0.0043	0.0005	0.0006	0.0027	-0.0044	-0.0034	0.0033	-0.0001	-0.0003	-0.0002	0.0006	0.0007	0.0003	0.0002	-0.0004	-0.0005	0.0007
L. amoi	2012	9	IMG1328b	Megachilidae	22.99	-0.0261	0.0600	0.0416	0.0178	-0.0096	-0.0165	-0.0066	-0.0017	0.0100	-0.0011	0.0039	-0.0066	-0.0054	-0.0148	0.0064	0.0016	0.0025	-0.0037	-0.0043	-0.0092	-0.0040	-0.0005	0.0015	-0.0001	0.0011	0.0002	0.0007	-0.0003	0.0002	-0.0004
L. amoi	2012	10	IMG1331b	Megachilidae	23.55	-0.0189	0.0484	0.0298	0.0263	-0.0093	-0.0299	-0.0190	-0.0062	0.0009	0.0039	-0.0043	-0.0033	-0.0062	-0.0178	0.0033	-0.0042	0.0011	0.0020	0.0008	-0.0025	-0.0001	0.0000	0.0014	0.0001	0.0001	-0.0002	0.0001	0.0000	0.0001	-0.0002
L. amoi	2012	11	IMG1334b	Megachilidae	22.50	-0.0169	0.0321	0.0038	0.0047	0.0401	0.0163	-0.0183	0.0236	-0.0062	-0.0009	-0.0099	0.0027	0.0031	-0.0190	0.0040	0.0054	0.0056	-0.0046	-0.0063	-0.0027	-0.0020	-0.0020	-0.0004	0.0007	-0.0020	0.0016	0.0018	-0.0005	0.0012	0.0002
L. amoi	2012	12	IMG1337b	Megachilidae	20.32	-0.0310	0.0455	0.0172	0.0218	-0.0163	-0.0032	-0.0102	0.0055	0.0050	-0.0192	-0.0057	-0.0044	-0.0038	-0.0045	-0.0044	0.0033	-0.0055	-0.0012	-0.0012	0.0016	-0.0017	0.0014	0.0012	-0.0003	0.0007	0.0005	0.0000	0.0001	-0.0005	0.0001
L. amoi	2012	13	IMG1340b	Megachilidae	21.26	-0.0572	0.0380	0.0148	0.0121	-0.0026	-0.0019	-0.0070	0.0114	-0.0153	-0.0100	0.0039	-0.0081	-0.0013	-0.0019	-0.0009	0.0013	-0.0030	-0.0045	0.0023	0.0026	-0.0002	0.0006	0.0001	-0.0008	-0.0002	0.0001	0.0001	-0.0001	0.0001	-0.0001
L. amoi	2012	14	IMG1343	Megachilidae	22.93	-0.0641	0.0682	0.0339	0.0368	0.0055	0.0062	-0.0007	0.0062	0.0012	-0.0024	0.0047	-0.0053	-0.0076	-0.0014	0.0028	0.0006	-0.0020	0.0052	0.0038	0.0000	0.0001	0.0016	0.0010	-0.0005	-0.0002	-0.0002	-0.0002	-0.0005	0.0002	0.0000
L. amoi	2012	15	IMG1346b	Megachilidae	23.00	-0.0201	0.0510	0.0313	0.0267	0.0087	0.0063	-0.0334	0.0148	0.0044	-0.0160	-0.0049	-0.0024	-0.0019	-0.0030	-0.0002	0.0016	0.0004	-0.0046	0.0001	0.0006	-0.0011	0.0003	0.0004	-0.0004	0.0000	0.0006	0.0001	0.0000	-0.0001	-0.0001
L. amoi	2012	16	IMG1349	Megachilidae	19.09	0.0487	0.0280	0.0473	0.0439	0.0112	0.0102	-0.0527	-0.0035	-0.0044	-0.0018	-0.0193	-0.0038	-0.0031	-0.0052	0.0062	0.0051	-0.0032	-0.0021	0.0003	0.0031	0.0020	0.0000	0.0006	0.0014	0.0005	-0.0003	0.0002	-0.0002	-0.0001	0.0001
L. amoi	2012	17	IMG1352	Megachilidae	25.02	0.0007	0.0664	0.0032	0.0243	-0.0133	-0.0086	0.0128	0.0147	-0.0070	0.0150	-0.0084	-0.0010	0.0030	-0.0137	-0.0010	0.0050	-0.0005	0.0019	0.0000	0.0009	0.0012	-0.0007	-0.0003	-0.0008	0.0003	0.0006	-0.0003	0.0001	0.0002	-0.0001
L. amoi	2012	18	IMG1355	Megachilidae	22.80	0.0073	0.0481	0.0444	0.0099	-0.0038	0.0022	-0.0207	-0.0085	-0.0071	0.0148	-0.0148	-0.0010	0.0054	-0.0006	-0.0004	-0.0069	0.0018	-0.0006	0.0003	0.0005	0.0014	0.0002	0.0012	0.0001	0.0006	0.0001	0.0002	-0.0001	0.0000	0.0000
L. amoi	2013	19	IMG2837	Megachilidae	22.36	-0.0403	-0.0357	-0.0119	-0.0020	-0.0362	0.0409	-0.0337	0.0162	-0.0077	0.0072	-0.0176	-0.0207	0.0088	0.0077	-0.0192	-0.0052	-0.0039	0.0060	-0.0017	0.0033	-0.0014	-0.0026	0.0006	-0.0006	-0.0007	-0.0021	0.0008	0.0002	0.0001	0.0001
L. amoi	2013	20	IMG2840	Megachilidae	23.41	-0.0745	0.0350	0.0311	-0.0279	0.0165	0.0111	-0.0367	0.0229	-0.0110	0.0061	0.0117	-0.0013	0.0088	-0.0010	-0.0028	0.0025	-0.0031	0.0002	0.0003	0.0008	-0.0012	0.0003	-0.0004	0.0011	0.0005	0.0001	0.0005	-0.0004	-0.0003	0.0001
L. amoi	2013	21	IMG2849	Megachilidae	23.05	0.0702	0.0070	0.0512	0.0257	-0.0064	0.0134	-0.0098	-0.0054	-0.0043	0.0258	-0.0132	-0.0109	0.0151	0.0080	0.0019	-0.0131	-0.0053	-0.0005	-0.0045	-0.0003	-0.0028	0.0009	0.0020	-0.0018	0.0010	-0.0002	0.0013	0.0008	0.0005	0.0004
L. amoi	2013	22	IMG2853	Megachilidae	24.19	-0.0278	0.0341	0.0416	-0.0442	0.0075	0.0000	-0.0014	0.0027	-0.0048	0.0040	0.0121	0.0058	0.0043	0.0083	-0.0001	0.0134	0.0071	-0.0017	-0.0018	-0.0043	-0.0010	-0.0015	0.0006	-0.0003	0.0002	0.0001	0.0002	0.0001	0.0006	-0.0002
L. amoi	2013	23	IMG2856	Megachilidae	24.63	-0.0478	0.0108	0.0255	-0.0299	0.0005	0.0076	-0.0492	-0.0078	-0.0186	-0.0035	-0.0198	-0.0100	0.0068	0.0062	0.0025	0.0041	-0.0032	0.0003	0.0018	0.0016	-0.0002	0.0008	0.0017	0.0000	-0.0003	0.0003	0.0001	0.0001	0.0000	0.0001
L. amoi	2013	24	IMG2860	Megachilidae	21.94	-0.1132	-0.0197	0.0408	0.0183	0.0027	-0.0235	-0.0213	-0.0011	-0.0200	0.0240	-0.0091	-0.0028	-0.0032	0.0024	-0.0034	-0.0044	0.0022	-0.0020	-0.0034	-0.0038	0.0005	-0.0013	0.0005	0.0010	-0.0009	-0.0002	-0.0004	0.0003	0.0000	0.0000
L. amoi	2013	25	IMG2863	Megachilidae	24.07	-0.0422	0.0099	0.0532	-0.0208	0.0069	-0.0018	-0.0108	0.0119	-0.0110	0.0212	0.0006	-0.0024	0.0005	0.0038	0.0000	0.0033	0.0036	0.0006	0.0029	-0.0018	0.0004	-0.0002	0.0006	0.0003	-0.0002	-0.0001	-0.0001	-0.0001	0.0002	-0.0001
L. amoi	2013	26	IMG2866	Megachilidae	23.59	0.0459	-0.0050	0.0106	0.0285	0.0114	0.0052	-0.0114	0.0191	-0.0031	0.0073	0.0010	-0.0021	0.0024	0.0005	0.0085	0.0106	0.0007	0.0004	-0.0012	-0.0006	0.0015	-0.0007	-0.0013	0.0000	0.0001	-0.0006	0.0002	-0.0002	0.0003	0.0003
L. amoi	2013	27	IMG2869	Megachilidae	23.36	0.0100	-0.0075	-0.0119	0.0243	0.0212	0.0087	0.0105	0.0279	0.0033	0.0109	0.0031	-0.0004	0.0131	0.0053	-0.0009	0.0078	-0.0018	0.0007	-0.0010	0.0014	-0.0016	0.0001	-0.0004	0.0003	0.0006	-0.0007	-0.0001	0.0001	0.0000	0.0000
L. anticaria	2010	1	IMG1774	*Anthophora*	22.99	-0.0139	-0.0451	0.0091	-0.0324	-0.0108	-0.0404	-0.0166	-0.0022	0.0134	0.0334	-0.0093	0.0033	-0.0073	0.0029	0.0042	-0.0012	0.0039	0.0017	-0.0021	0.0009	0.0006	0.0012	0.0006	0.0006	-0.0009	0.0000	0.0001	0.0003	-0.0001	-0.0001
L. anticaria	2010	2	IMG1785	*Anthophora*	26.03	0.0495	-0.0423	0.0239	-0.0328	0.0176	-0.0267	0.0196	-0.0084	0.0088	0.0184	0.0000	0.0205	0.0028	-0.0112	-0.0099	0.0043	0.0023	-0.0008	-0.0025	-0.0018	0.0015	0.0017	-0.0007	0.0002	0.0000	0.0000	0.0005	0.0005	0.0003	-0.0002
L. anticaria	2010	3	IMG1788	*Anthophora*	24.35	0.0147	-0.0170	0.0010	-0.0157	-0.0031	-0.0054	0.0106	0.0038	0.0184	0.0208	0.0132	0.0158	-0.0020	-0.0074	-0.0033	0.0012	0.0008	-0.0018	-0.0035	0.0006	0.0000	0.0039	-0.0004	0.0011	-0.0005	0.0008	0.0003	-0.0001	-0.0001	0.0003
L. anticaria	2010	4	IMG1794	*Anthophora*	23.93	0.0595	-0.0642	-0.0321	0.0054	0.0010	-0.0092	-0.0043	-0.0008	0.0116	0.0075	-0.0029	0.0042	0.0164	0.0049	0.0086	-0.0005	-0.0058	0.0005	0.0030	-0.0008	-0.0009	0.0012	0.0002	0.0013	-0.0005	0.0012	0.0005	0.0000	0.0002	-0.0003
L. anticaria	2010	5	IMG1805	*Anthophora*	26.74	0.2208	-0.0310	0.0249	0.0095	-0.0168	-0.0155	-0.0176	-0.0282	-0.0213	-0.0111	0.0073	0.0073	0.0024	-0.0088	-0.0080	0.0016	-0.0009	-0.0022	0.0006	0.0036	-0.0017	0.0003	0.0008	-0.0001	0.0006	0.0005	-0.0007	0.0001	-0.0004	-0.0005
L. anticaria	2010	6	IMG1808	*Anthophora*	26.37	-0.0240	-0.0251	-0.0333	-0.0435	-0.0135	-0.0129	0.0208	0.0081	0.0216	0.0186	0.0037	0.0077	0.0004	-0.0026	0.0063	0.0000	0.0086	0.0034	0.0004	0.0009	-0.0002	0.0013	-0.0004	-0.0001	-0.0005	0.0001	-0.0003	-0.0001	0.0001	0.0001
L. anticaria	2010	7	IMG1815	*Anthophora*	23.53	0.1225	-0.0441	0.0279	0.0112	-0.0065	0.0191	0.0015	0.0021	-0.0078	0.0062	-0.0057	0.0035	0.0009	0.0089	-0.0048	-0.0002	0.0056	0.0023	-0.0037	-0.0031	-0.0008	-0.0002	0.0020	0.0005	-0.0005	-0.0008	-0.0004	-0.0011	0.0005	0.0002
L. anticaria	2010	8	IMG1820	*Anthophora*	22.62	0.1592	-0.0963	0.0256	0.0066	0.0056	0.0309	-0.0020	-0.0037	-0.0126	-0.0073	-0.0034	0.0152	0.0033	0.0127	-0.0090	0.0051	0.0066	0.0037	-0.0017	-0.0045	-0.0044	-0.0010	0.0027	0.0006	0.0000	-0.0023	-0.0007	-0.0010	0.0008	0.0003
L. anticaria	2010	9	IMG1827	*Anthophora*	23.98	0.0870	-0.0456	0.0116	-0.0194	-0.0159	0.0137	-0.0066	-0.0007	0.0029	-0.0069	0.0014	-0.0022	0.0023	-0.0016	0.0025	0.0034	0.0086	0.0009	0.0010	-0.0017	0.0022	-0.0012	0.0009	-0.0009	-0.0004	-0.0003	0.0003	-0.0003	0.0001	0.0001
L. anticaria	2010	10	IMG1833	*Anthophora*	24.25	0.0571	-0.0447	0.0336	-0.0511	-0.0334	-0.0060	-0.0040	-0.0016	-0.0072	-0.0072	0.0077	0.0033	0.0078	0.0043	-0.0032	-0.0007	0.0027	0.0030	-0.0024	-0.0003	0.0009	0.0004	0.0005	0.0007	0.0004	0.0003	-0.0001	-0.0002	0.0002	0.0001
L. anticaria	2010	11	IMG1836	*Anthophora*	23.73	0.0909	-0.0706	-0.0390	-0.0682	0.0102	-0.0062	-0.0124	0.0081	-0.0064	0.0017	0.0007	0.0096	0.0101	-0.0020	0.0094	0.0012	-0.0016	0.0002	0.0029	-0.0013	0.0013	-0.0013	0.0008	-0.0010	0.0015	0.0000	-0.0003	0.0002	0.0000	-0.0002

L. anticaria	2010	12	IMG1839	*Anthophora*	24.10	0.0708	-0.0731	-0.0335	-0.0763	-0.0266	-0.0136	-0.0161	-0.0062	0.0014	0.0027	0.0134	0.0053	0.0091	-0.0075	0.0047	0.0028	-0.0005	0.0031	0.0002	-0.0008	0.0019	0.0006	-0.0004	-0.0003	0.0001	0.0010	-0.0001	0.0001	0.0001	0.0000
L. anticaria	2010	13	P3260478	Megachilidae	23.15	0.0407	0.0450	0.0366	0.0270	-0.0003	0.0036	-0.0060	0.0007	0.0037	0.0205	-0.0165	0.0035	-0.0023	0.0040	0.0067	0.0040	-0.0075	-0.0029	-0.0062	0.0014	-0.0001	0.0003	-0.0005	0.0008	0.0008	0.0003	0.0000	0.0002	-0.0001	0.0002
L. anticaria	2010	14	P3260482	Megachilidae	20.28	-0.0172	-0.0507	0.0766	0.0134	0.0017	-0.0136	0.0289	0.0135	0.0080	0.0287	0.0036	0.0044	-0.0128	-0.0052	0.0077	0.0013	-0.0083	-0.0021	0.0047	-0.0001	0.0000	-0.0005	-0.0015	-0.0011	-0.0012	0.0002	-0.0003	-0.0002	0.0001	0.0003
L. anticaria	2010	15	P3260489	Megachilidae	20.29	-0.0160	0.0246	0.0511	-0.0325	-0.0223	-0.0273	0.0058	0.0017	0.0078	0.0046	0.0071	0.0040	-0.0058	0.0015	0.0014	-0.0023	-0.0003	0.0019	0.0012	-0.0004	0.0002	-0.0005	-0.0003	-0.0004	-0.0001	-0.0002	-0.0001	-0.0001	0.0000	0.0003
L. anticaria	2010	16	IMG1855	*Anthophora*	22.96	-0.0391	-0.1068	0.0226	-0.0338	-0.0087	0.0103	0.0154	0.0174	-0.0018	0.0117	0.0051	-0.0020	0.0129	-0.0034	0.0040	-0.0009	0.0036	-0.0038	-0.0033	0.0002	0.0010	0.0001	0.0000	0.0012	-0.0004	-0.0002	0.0001	0.0001	0.0001	-0.0001
L. anticaria	2012	17	IMG1277	*Anthophora*	22.61	0.0785	0.0035	-0.0005	0.0411	-0.0078	-0.0145	0.0255	0.0098	0.0157	0.0045	-0.0079	0.0060	0.0053	0.0017	-0.0079	0.0056	-0.0016	0.0019	-0.0039	0.0027	-0.0003	-0.0001	-0.0002	0.0017	0.0007	0.0006	-0.0004	0.0000	0.0001	0.0001
L. anticaria	2012	18	IMG1280	*Anthophora*	21.33	0.0546	-0.0740	0.0057	0.0526	0.0081	-0.0150	0.0284	0.0167	0.0147	0.0027	-0.0085	0.0052	0.0029	0.0059	0.0073	0.0017	0.0016	0.0001	0.0005	0.0006	0.0016	-0.0001	0.0012	0.0002	0.0007	-0.0008	-0.0003	-0.0001	0.0000	0.0000
L. anticaria	2012	19	IMG1283	*Anthophora*	20.27	0.0354	-0.0596	0.0175	0.0202	-0.0228	-0.0012	0.0085	0.0075	0.0142	0.0153	-0.0086	-0.0028	0.0083	-0.0027	-0.0025	0.0044	0.0013	0.0019	-0.0004	0.0022	0.0005	0.0005	0.0000	-0.0005	-0.0007	-0.0001	-0.0002	0.0003	0.0003	0.0000
L. anticaria	2012	20	IMG1285	*Anthophora*	21.01	-0.0151	-0.0517	0.0009	0.0564	-0.0210	0.0058	0.0444	0.0255	0.0114	-0.0042	-0.0010	0.0021	0.0063	-0.0032	0.0018	-0.0002	-0.0022	0.0058	-0.0027	0.0032	0.0017	0.0011	0.0000	-0.0006	-0.0005	-0.0007	0.0002	-0.0002	0.0001	0.0000
L. anticaria	2012	21	IMG1288	*Anthophora*	25.18	-0.0473	-0.0253	0.0083	-0.0132	-0.0056	-0.0093	-0.0027	0.0170	0.0086	-0.0013	-0.0094	-0.0004	0.0118	0.0027	0.0037	0.0040	-0.0036	-0.0067	0.0005	0.0015	-0.0008	-0.0014	0.0010	0.0002	-0.0008	0.0010	-0.0004	0.0003	0.0000	0.0001
L. anticaria	2012	22	IMG1291	*Anthophora*	21.90	0.1457	-0.0477	0.0745	0.0215	-0.0162	-0.0024	0.0039	-0.0093	-0.0122	-0.0090	0.0017	0.0136	-0.0026	-0.0003	-0.0020	0.0040	-0.0019	-0.0016	0.0027	0.0008	-0.0022	-0.0036	0.0012	0.0001	0.0010	0.0002	0.0000	0.0009	-0.0007	-0.0001
L. anticaria	2012	23	IMG1294	*Anthophora*	21.73	0.0269	-0.0400	0.0351	0.0029	-0.0157	-0.0158	0.0044	0.0021	0.0084	0.0036	-0.0028	0.0020	0.0036	-0.0021	-0.0028	0.0014	-0.0017	0.0024	-0.0010	0.0026	0.0008	0.0009	0.0003	0.0008	-0.0002	0.0002	-0.0003	0.0000	0.0001	0.0001
L. anticaria	2012	24	IMG1297	*Anthophora*	19.94	0.0209	-0.0871	0.0139	0.0110	-0.0016	0.0005	-0.0037	0.0454	0.0301	-0.0110	-0.0074	0.0055	0.0080	-0.0047	-0.0034	0.0047	0.0026	0.0011	-0.0062	0.0058	0.0000	-0.0008	-0.0012	0.0006	0.0001	-0.0005	0.0002	-0.0003	0.0000	-0.0001
L. anticaria	2012	25	IMG1300	*Anthophora*	19.67	0.0449	-0.0750	0.0500	-0.0054	0.0132	0.0013	-0.0048	0.0434	-0.0212	-0.0062	0.0070	-0.0004	0.0136	-0.0099	0.0009	-0.0003	0.0005	0.0025	0.0046	0.0038	0.0008	-0.0013	0.0003	-0.0016	0.0007	0.0002	-0.0001	0.0005	0.0000	-0.0005
L. anticaria	2012	26	IMG1310	*Anthophora*	21.94	0.1064	-0.0604	0.0249	0.0320	-0.0121	0.0155	-0.0205	0.0110	0.0036	-0.0010	0.0068	-0.0004	0.0160	-0.0078	-0.0043	0.0034	0.0037	-0.0013	-0.0020	0.0043	0.0000	-0.0003	0.0002	-0.0009	0.0007	0.0000	-0.0002	-0.0001	0.0004	0.0000
L. depauperata	2010	1	IMG0312	Megachilidae	22.85	0.0471	0.0386	0.0261	0.0244	-0.0197	0.0222	0.0069	-0.0063	0.0105	0.0012	-0.0217	-0.0033	-0.0062	-0.0033	0.0079	0.0092	-0.0071	-0.0052	-0.0007	-0.0009	-0.0008	-0.0019	0.0004	0.0003	-0.0002	0.0014	0.0001	0.0015	-0.0005	0.0004
L. depauperata	2010	2	IMG0327	Megachilidae	22.73	-0.0542	0.0015	0.0971	0.0187	-0.0177	0.0178	0.0431	0.0087	0.0013	0.0055	0.0062	-0.0025	-0.0120	0.0051	-0.0005	-0.0029	0.0050	0.0015	0.0000	-0.0034	-0.0004	0.0016	0.0003	0.0002	-0.0009	-0.0005	-0.0001	-0.0005	0.0006	-0.0004
L. depauperata	2010	3	IMG0341	Megachilidae	21.56	0.0942	0.0166	0.0482	0.0060	-0.0482	-0.0393	0.0228	-0.0219	0.0033	-0.0113	-0.0094	0.0035	-0.0016	-0.0031	-0.0027	0.0043	0.0002	-0.0016	-0.0007	0.0036	0.0002	-0.0029	0.0011	0.0037	0.0018	-0.0003	0.0005	0.0004	-0.0007	0.0002
L. depauperata	2010	4	IMG0349	Megachilidae	22.10	-0.0110	0.0011	0.0484	-0.0202	-0.0032	-0.0199	-0.0099	-0.0022	-0.0061	0.0072	0.0028	-0.0040	-0.0038	-0.0128	-0.0037	0.0024	-0.0007	-0.0010	0.0024	0.0009	-0.0010	0.0006	0.0002	0.0010	-0.0007	0.0002	0.0006	-0.0001	0.0001	-0.0002
L. depauperata	2010	5	P3260426	Megachilidae	21.61	-0.0199	0.0005	0.0560	-0.0268	-0.0071	-0.0241	-0.0121	0.0088	-0.0108	-0.0019	-0.0065	-0.0034	-0.0096	0.0033	0.0041	0.0014	-0.0014	0.0057	0.0046	0.0010	0.0010	0.0013	-0.0005	0.0005	-0.0004	-0.0005	-0.0001	-0.0001	0.0003	0.0000
L. depauperata	2010	6	P3260432	Megachilidae	24.15	0.0742	0.0626	0.0043	-0.0195	-0.0179	0.0290	0.0104	0.0085	-0.0093	-0.0325	-0.0085	-0.0045	-0.0084	-0.0084	-0.0017	0.0000	0.0018	-0.0010	0.0033	-0.0021	0.0001	-0.0008	-0.0013	0.0002	0.0006	0.0009	0.0011	0.0005	-0.0004	0.0000
L. depauperata	2010	7	P3260440	Megachilidae	20.53	0.1168	-0.0292	0.0531	0.0104	-0.0236	-0.0136	-0.0219	-0.0091	-0.0142	-0.0036	0.0055	-0.0043	-0.0232	0.0011	0.0041	0.0018	-0.0011	0.0003	-0.0028	-0.0054	0.0042	-0.0013	-0.0037	0.0017	0.0000	0.0003	0.0005	0.0001	0.0000	0.0002
L. depauperata	2010	8	P3260455	Megachilidae	22.43	0.0653	0.0069	0.0144	0.0038	0.0146	-0.0022	0.0030	-0.0057	-0.0015	-0.0089	-0.0004	0.0041	0.0025	0.0058	0.0077	0.0038	-0.0025	0.0082	0.0013	0.0007	-0.0009	0.0011	0.0001	0.0009	0.0001	-0.0017	-0.0003	-0.0001	0.0005	0.0002
L. depauperata	2010	9	P3260461	Megachilidae	22.07	0.0665	0.0155	0.0814	-0.0165	-0.0226	0.0009	0.0004	-0.0228	-0.0080	-0.0063	0.0090	-0.0029	-0.0122	-0.0117	-0.0027	0.0004	0.0027	-0.0005	0.0043	-0.0024	-0.0036	0.0012	-0.0018	0.0017	-0.0018	-0.0014	0.0016	0.0000	-0.0002	-0.0004
L. depauperata	2010	10	P3260464	Megachilidae	22.07	0.0595	-0.0090	0.0521	-0.0125	-0.0038	0.0184	0.0138	-0.0046	-0.0077	0.0028	-0.0053	0.0122	-0.0127	0.0038	0.0055	0.0031	-0.0003	0.0014	0.0027	-0.0019	0.0006	0.0000	-0.0014	0.0004	-0.0002	-0.0006	-0.0002	0.0004	0.0003	-0.0002
L. depauperata	2010	11	P3260474	Megachilidae	23.66	0.0732	-0.0046	0.0223	-0.0111	0.0053	-0.0322	0.0220	-0.0086	-0.0212	0.0157	-0.0097	0.0013	0.0014	0.0011	0.0089	0.0034	-0.0045	-0.0056	0.0020	-0.0017	-0.0019	-0.0009	0.0010	-0.0010	0.0016	-0.0001	0.0002	-0.0004	-0.0011	0.0002
L. depauperata	2010	12	P3260477	Megachilidae	22.66	0.0663	0.0488	0.0043	0.0028	-0.0213	0.0071	-0.0013	-0.0144	0.0002	0.0055	-0.0151	-0.0029	-0.0022	0.0003	0.0037	0.0040	-0.0025	0.0001	-0.0005	0.0011	0.0006	0.0000	-0.0005	0.0004	0.0004	0.0002	0.0001	0.0003	0.0000	0.0002
L. depauperata	2013	16	IMG2771	Megachilidae	23.59	0.0841	0.0740	0.0015	0.0259	0.0094	0.0060	-0.0151	0.0038	0.0087	-0.0273	0.0091	0.0043	0.0001	-0.0005	-0.0076	-0.0014	0.0001	0.0003	0.0035	0.0033	-0.0002	0.0007	0.0002	0.0010	0.0006	-0.0005	-0.0002	0.0000	-0.0003	-0.0003
L. depauperata	2013	17	IMG2763	Megachilidae	25.42	-0.0314	0.0347	0.0738	-0.0471	-0.0061	-0.0038	0.0151	0.0007	-0.0237	0.0076	-0.0041	0.0013	-0.0008	0.0185	0.0047	0.0020	-0.0028	0.0025	-0.0063	0.0006	-0.0029	0.0026	-0.0013	0.0011	0.0003	0.0008	-0.0007	0.0005	-0.0003	-0.0006
L. depauperata	2013	18	IMG2758	Megachilidae	21.58	-0.0139	0.0458	0.0058	-0.0535	-0.0072	-0.0130	0.0072	0.0025	0.0009	-0.0268	0.0027	0.0005	0.0020	0.0042	0.0010	-0.0012	-0.0004	0.0007	0.0021	0.0013	-0.0010	0.0005	0.0003	-0.0016	-0.0007	0.0004	-0.0002	0.0001	-0.0001	0.0002
L. depauperata	2013	19	IMG2755	Megachilidae	23.53	0.1256	-0.0165	0.0133	0.0663	0.0129	-0.0161	-0.0142	-0.0241	0.0229	0.0088	0.0008	-0.0047	-0.0050	0.0019	-0.0049	-0.0001	0.0054	-0.0067	0.0025	-0.0020	-0.0013	0.0017	-0.0005	-0.0031	-0.0031	-0.0003	0.0008	0.0006	-0.0008	-0.0003
L. depauperata	2013	20	IMG2750	Megachilidae	21.02	0.0390	0.0580	0.0641	-0.0058	0.0021	0.0062	0.0015	0.0122	-0.0276	0.0015	-0.0172	0.0031	-0.0067	0.0113	0.0014	-0.0047	-0.0018	-0.0014	0.0035	0.0017	-0.0003	0.0012	-0.0006	0.0010	0.0016	0.0004	-0.0001	0.0007	-0.0005	0.0000
L. depauperata	2013	21	IMG2741	Megachilidae	21.96	0.0729	0.0272	0.0360	0.0141	0.0157	0.0284	-0.0122	-0.0064	0.0047	-0.0329	-0.0010	0.0049	0.0103	-0.0019	0.0091	-0.0023	-0.0016	0.0007	0.0004	0.0009	0.0014	-0.0006	0.0021	-0.0011	0.0004	0.0004	0.0008	0.0002	-0.0001	0.0002
L. depauperata	2013	22	IMG2734	Megachilidae	20.27	-0.1641	0.1116	0.0122	-0.0506	0.0054	0.0052	-0.0179	0.0122	-0.0019	0.0015	0.0024	-0.0052	0.0095	0.0113	-0.0069	0.0019	-0.0063	0.0061	-0.0010	-0.0021	-0.0013	0.0012	0.0007	-0.0002	-0.0022	0.0023	0.0008	-0.0003	-0.0017	0.0001
L. depauperata	2013	23	IMG2728	Megachilidae	21.40	-0.0469	0.1042	0.0087	-0.0396	0.0164	-0.0475	-0.0255	-0.0038	-0.0097	0.0017	-0.0029	0.0007	-0.0039	0.0165	-0.0037	0.0072	0.0022	-0.0024	-0.0077	0.0024	0.0012	0.0014	-0.0004	-0.0015	-0.0005	-0.0006	-0.0003	0.0001	-0.0001	-0.0005
L. depauperata	2013	24	IMG2721	Megachilidae	23.02	-0.0168	0.0741	0.0062	-0.0026	0.0160	-0.0337	0.0051	0.0013	-0.0125	-0.0220	0.0024	-0.0028	0.0010	0.0013	-0.0029	-0.0004	0.0029	0.0010	-0.0045	0.0009	-0.0015	0.0003	-0.0006	-0.0005	0.0000	0.0003	0.0003	-0.0003	-0.0001	0.0001
L. depauperata	2013	25	IMG2709	Megachilidae	22.72	0.0445	0.0549	0.0500	-0.0133	-0.0025	-0.0094	0.0149	-0.0062	-0.0116	-0.0284	0.0008	0.0015	-0.0002	0.0042	0.0005	0.0060	0.0048	-0.0001	0.0017	0.0016	-0.0043	-0.0003	-0.0004	0.0005	-0.0001	-0.0003	0.0008	0.0000	-0.0001	0.0002
L. lilacina	2011	1	IMG4818	*Anthophora*	20.76	0.1177	-0.0384	-0.0156	-0.0013	0.0001	-0.0445	-0.0284	-0.0188	0.0095	0.0019	0.0129	0.0004	-0.0032	0.0023	-0.0023	0.0018	-0.0052	-0.0032	0.0002	0.0003	-0.0028	0.0018	-0.0002	-0.0009	-0.0008	0.0007	0.0001	0.0004	0.0001	-0.0003
L. lilacina	2011	2	IMG4821	*Anthophora*	18.51	0.0235	-0.0776	-0.0584	-0.0324	-0.0263	-0.0130	-0.0220	0.0090	0.0115	0.0141	0.0013	-0.0094	-0.0032	0.0066	-0.0042	-0.0036	-0.0021	-0.0014	0.0035	0.0038	-0.0046	0.0015	-0.0010	-0.0001	0.0010	0.0012	0.0009	-0.0004	0.0008	-0.0006
L. lilacina	2011	3	IMG4825	*Anthophora*	21.37	0.0432	-0.0071	-0.0346	0.0144	0.0097	-0.0009	-0.0177	0.0059	0.0262	-0.0012	0.0094	0.0049	0.0060	0.0013	-0.0114	-0.0031	0.0050	-0.0026	-0.0007	-0.0005	-0.0001	0.0004	-0.0009	0.0010	0.0002	0.0001	0.0002	0.0000	-0.0002	-0.0002
L. lilacina	2011	4	IMG4829	*Anthophora*	21.86	0.0099	0.0176	-0.0074	-0.0224	0.0107	-0.0123	-0.0221	0.0037	0.0200	0.0098	0.0013	0.0039	-0.0089	0.0104	-0.0042	-0.0024	0.0032	0.0040	0.0029	-0.0009	-0.0006	-0.0002	0.0009	0.0009	-0.0008	0.0001	-0.0001	0.0003	0.0000	0.0002
L. lilacina	2011	5	IMG4835	*Anthophora*	22.23	0.0594	-0.0356	-0.0036	-0.0161	-0.0102	0.0214	0.0015	0.0084	0.0104	0.0029	0.0052	0.0018	-0.0047	0.0152	-0.0018	-0.0016	0.0035	-0.0043	-0.0016	-0.0009	0.0007	-0.0007	0.0003	-0.0007	0.0000	-0.0004	-0.0004	-0.0002	-0.0001	-0.0002
L. lilacina	2011	6	IMG4838	*Anthophora*	21.51	0.0221	-0.0381	0.0036	-0.0164	-0.0055	-0.0090	-0.0216	-0.0156	-0.0002	0.0072	0.0045	-0.0076	-0.0050	0.0057	0.0003	-0.0057	-0.0026	-0.0023	-0.0050	-0.0004	-0.0008	0.0011	0.0005	0.0006	-0.0007	-0.0001	0.0003	0.0001	-0.0002	0.0000
L. lilacina	2011	7	IMG4841	*Anthophora*	21.10	-0.0411	-0.0434	-0.0241	-0.0079	-0.0002	-0.0178	-0.0268	0.0191	0.0123	0.0150	-0.0004	-0.0008	-0.0070	0.0045	-0.0008	-0.0025	0.0009	-0.0013	0.0000	0.0020	-0.0004	-0.0002	0.0001	0.0007	-0.0002	0.0003	-0.0002	-0.0001	-0.0001	-0.0002
L. lilacina	2011	8	IMG4847	*Anthophora*	20.05	0.0784	-0.0535	-0.0238	0.0181	0.0025	-0.0071	-0.0092	0.0081	0.0225	-0.0047	0.0076	0.0069	0.0042	0.0038	0.0045	0.0009	0.0021	0.0002	-0.0029	0.0002	0.0011	0.0008	0.0003	0.0006	0.0000	-0.0003	0.0001	0.0001	0.0000	-0.0001
L. lilacina	2011	9	IMG4850	*Anthophora*	21.48	0.0136	-0.0416	-0.0037	-0.0403	-0.0058	0.0087	-0.0478	0.0137	0.0077	0.0089	0.0027	-0.0009	-0.0021	-0.0039	0.0030	0.0027	-0.0009	0.0020	0.0007	0.0023	0.0022	0.0007	-0.0012	0.0002	0.0004	0.0000	-0.0002	-0.0003	0.0001	-0.0002
L. lilacina	2011	10	IMG4853	*Anthophora*	19.58	0.1001	-0.0692	0.0056	-0.0090	0.0490	0.0089	-0.0246	-0.0018	-0.0072	0.0273	-0.0158	0.0054	0.0005	-0.0104	0.0073	0.0044	-0.0014	0.0017	-0.0014	-0.0054	-0.0022	-0.0003	0.0018	-0.0016	-0.0007	-0.0001	0.0009	0.0002	0.0008	0.0001
L. lilacina	2011	11	IMG4857	*Anthophora*	22.37	0.0326	-0.0477	-0.0211	0.0218	0.0151	0.0180	-0.0039	0.0089	-0.0010	0.0125	-0.0017	0.0006	0.0065	-0.0092	0.0017	-0.0050	0.0043	0.0009	0.0005	-0.0013	0.0020	0.0000	0.0009	-0.0003	-0.0011	-0.0002	-0.0005	0.0003	0.0002	-0.0002
L. lilacina	2011	12	IMG4860	*Anthophora*	20.87	0.1026	-0.0508	-0.0397	0.0295	0.0325	0.0124	-0.0181	0.0031	0.0273	0.0063	-0.0052	0.0055	0.0093	0.0122	-0.0012	-0.0042	-0.0014	0.0020	-0.0006	-0.0030	-0.0033	0.0026	0.0004	-0.0005	0.0014	-0.0016	0.0004	0.0004	0.0006	-0.0003

L. lilacina	2011	13	IMG4863	*Anthophora*	24.48	0.0958	-0.0122	-0.0644	-0.0019	0.0098	0.0017	0.0063	0.0088	-0.0012	0.0054	0.0076	0.0026	0.0080	-0.0017	-0.0010	0.0054	0.0011	-0.0039	-0.0003	-0.0005	-0.0015	-0.0011	-0.0004	-0.0013	0.0005	0.0005	-0.0004	0.0001	-0.0002	-0.0002
L. lilacina	2011	14	IMG4867	*Anthophora*	22.07	0.0342	-0.0479	-0.0062	-0.0019	0.0186	-0.0214	0.0194	0.0030	-0.0026	0.0171	-0.0070	0.0030	0.0048	0.0034	-0.0011	-0.0031	-0.0006	-0.0002	-0.0008	-0.0011	-0.0012	0.0008	0.0007	0.0004	0.0004	-0.0001	-0.0001	0.0001	-0.0002	-0.0001
L. lilacina	2011	15	IMG4870	*Anthophora*	19.88	0.0804	0.0381	0.0143	0.0257	-0.0158	0.0069	0.0130	-0.0265	0.0237	-0.0024	0.0008	0.0015	-0.0063	0.0019	0.0003	-0.0053	0.0042	-0.0020	0.0013	-0.0016	-0.0001	0.0000	0.0010	-0.0030	-0.0012	-0.0006	0.0006	0.0001	-0.0003	-0.0001
L. lilacina	2011	16	IMG4873	*Anthophora*	21.15	0.0464	-0.0083	-0.0179	0.0039	-0.0238	-0.0334	-0.0162	-0.0094	0.0029	-0.0096	0.0061	0.0011	0.0084	-0.0061	-0.0020	-0.0020	0.0009	0.0014	-0.0051	0.0006	0.0014	-0.0001	0.0002	0.0009	0.0007	0.0003	-0.0001	-0.0002	0.0000	0.0002
L. lilacina	2011	17	IMG4876	*Anthophora*	20.94	0.1194	-0.0609	-0.0309	0.0116	-0.0134	-0.0312	-0.0095	-0.0094	-0.0045	0.0068	0.0004	-0.0056	0.0144	0.0014	0.0061	-0.0010	0.0037	-0.0027	-0.0007	-0.0028	-0.0021	-0.0003	0.0005	-0.0019	0.0008	0.0000	0.0008	-0.0005	0.0000	0.0011
L. lilacina	2011	18	IMG4880	*Anthophora*	22.14	-0.0900	-0.0863	-0.0443	-0.0487	0.0191	-0.0214	0.0037	0.0136	-0.0108	0.0433	-0.0073	0.0051	0.0050	-0.0019	0.0025	0.0011	-0.0048	0.0008	0.0067	-0.0024	-0.0021	0.0017	0.0019	0.0015	0.0009	0.0011	-0.0010	-0.0007	-0.0008	0.0000
L. lilacina	2011	19	IMG4884	*Anthophora*	21.27	-0.0207	-0.0898	-0.0265	0.0259	-0.0152	-0.0296	-0.0102	-0.0137	-0.0025	0.0156	-0.0027	0.0175	0.0018	-0.0080	0.0047	0.0008	0.0034	-0.0013	0.0003	0.0017	0.0032	0.0032	0.0005	0.0015	-0.0008	0.0005	-0.0004	0.0005	-0.0004	-0.0004
L. lilacina	2011	20	IMG4844	*Anthophora*	19.32	-0.1246	-0.1444	-0.0130	0.0793	-0.0765	-0.0285	0.0075	-0.0155	-0.0092	0.0048	-0.0106	0.0423	-0.0107	0.0178	0.0054	-0.0002	0.0032	-0.0018	0.0076	0.0002	-0.0032	-0.0029	-0.0015	0.0022	-0.0005	0.0019	0.0008	-0.0009	0.0007	0.0000
L. lilacina	2012	21	IMG1132	*Anthophora*	18.32	-0.1727	-0.1552	-0.0190	0.0052	0.0271	0.0091	-0.0393	0.0045	-0.0312	-0.0206	-0.0227	0.0269	-0.0095	0.0100	-0.0109	-0.0038	0.0009	0.0003	0.0047	-0.0038	-0.0034	-0.0006	-0.0025	-0.0027	0.0002	0.0005	-0.0011	0.0011	0.0004	0.0003
L. lilacina	2012	22	IMG1135	*Anthophora*	21.51	-0.0640	-0.0499	-0.0169	0.0258	-0.0044	-0.0034	-0.0141	0.0090	0.0201	-0.0319	-0.0149	-0.0017	0.0136	0.0083	0.0027	-0.0004	-0.0015	-0.0018	0.0018	-0.0008	0.0020	0.0000	-0.0001	0.0012	-0.0009	0.0001	-0.0006	0.0003	-0.0001	-0.0001
L. lilacina	2012	23	IMG1138	*Anthophora*	20.05	-0.1204	-0.0797	-0.0426	0.0346	0.0172	-0.0213	0.0054	0.0257	-0.0079	-0.0098	-0.0069	0.0122	0.0110	0.0044	0.0005	-0.0006	-0.0032	-0.0026	0.0045	-0.0003	-0.0015	-0.0007	-0.0022	0.0006	-0.0009	-0.0001	-0.0013	-0.0007	0.0003	-0.0001
L. lilacina	2012	24	IMG1141	*Anthophora*	19.33	-0.1279	-0.0951	-0.0399	0.0107	0.0019	-0.0104	0.0020	0.0042	-0.0159	-0.0355	0.0025	0.0188	0.0088	0.0166	-0.0057	-0.0007	-0.0078	0.0032	-0.0016	-0.0010	-0.0013	-0.0008	-0.0026	-0.0024	-0.0016	-0.0019	0.0018	-0.0003	-0.0009	-0.0005
L. lilacina	2012	25	IMG1144	*Anthophora*	19.19	-0.1095	-0.0737	0.0198	0.0579	-0.0030	-0.0553	0.0172	0.0123	0.0073	0.0009	-0.0057	0.0047	-0.0100	0.0173	0.0000	-0.0011	-0.0010	0.0062	-0.0014	0.0008	0.0042	-0.0015	0.0015	-0.0010	-0.0004	0.0009	0.0007	0.0002	0.0002	-0.0001
L. platycalyx	2010	1	IMG0631	*Anthophora*	23.58	-0.0073	-0.0239	0.0085	-0.0384	-0.0178	0.0117	0.0092	0.0299	0.0102	0.0189	-0.0056	-0.0014	-0.0132	0.0044	-0.0035	-0.0051	0.0028	-0.0011	0.0016	-0.0011	0.0016	-0.0015	-0.0016	-0.0008	-0.0005	-0.0002	-0.0005	-0.0007	0.0000	0.0004
L. platycalyx	2010	2	IMG0640	*Anthophora*	23.66	0.0543	-0.0236	-0.0073	-0.0274	-0.0044	0.0288	0.0225	0.0000	-0.0017	0.0093	-0.0039	0.0019	0.0023	-0.0051	-0.0025	0.0034	-0.0011	-0.0024	0.0017	0.0021	-0.0001	0.0004	-0.0005	-0.0005	-0.0009	0.0002	-0.0001	0.0004	-0.0002	0.0000
L. platycalyx	2010	3	IMG0642	*Anthophora*	23.90	0.0400	-0.0238	0.0131	-0.0173	0.0015	0.0161	0.0290	0.0064	-0.0081	0.0142	0.0002	-0.0017	0.0023	-0.0058	-0.0072	0.0018	0.0041	-0.0015	0.0020	0.0002	0.0001	0.0000	0.0003	-0.0004	-0.0014	-0.0005	0.0001	-0.0002	-0.0001	-0.0003
L. platycalyx	2010	4	IMG0672	*Anthophora*	22.58	-0.0710	0.0040	0.0316	-0.0924	-0.0197	-0.0182	0.0030	-0.0150	0.0094	0.0193	0.0067	0.0025	0.0100	0.0053	0.0004	-0.0017	-0.0035	0.0006	-0.0003	-0.0002	0.0012	-0.0025	0.0005	0.0003	-0.0005	-0.0003	-0.0003	-0.0002	-0.0003	0.0003
L. platycalyx	2010	5	IMG0676	*Anthophora*	26.10	-0.0370	0.0207	-0.0348	-0.0531	-0.0261	-0.0234	-0.0094	-0.0052	0.0188	0.0038	0.0076	-0.0030	0.0000	-0.0083	-0.0005	0.0003	0.0007	0.0003	0.0014	-0.0021	-0.0012	0.0001	0.0005	-0.0001	0.0005	-0.0003	-0.0005	0.0000	-0.0003	0.0002
L. platycalyx	2010	6	IMG0680	*Anthophora*	22.71	-0.0437	0.0427	0.0041	-0.0723	0.0071	0.0205	0.0061	-0.0061	0.0177	-0.0045	0.0024	0.0039	-0.0066	-0.0035	0.0052	-0.0005	0.0003	0.0013	-0.0006	-0.0010	-0.0015	-0.0006	0.0010	-0.0006	0.0000	-0.0008	-0.0001	0.0000	0.0001	-0.0001
L. platycalyx	2010	7	IMG0688	*Anthophora*	22.72	0.0483	-0.0163	0.0363	-0.0282	-0.0372	-0.0049	0.0161	-0.0201	0.0052	0.0105	0.0025	0.0008	-0.0009	-0.0073	0.0033	-0.0008	-0.0048	0.0017	0.0040	0.0009	0.0004	0.0008	-0.0011	-0.0018	-0.0013	0.0000	-0.0002	0.0006	-0.0001	0.0003
L. platycalyx	2010	8	IMG0692	*Anthophora*	19.37	0.0246	-0.0213	-0.0056	-0.0265	-0.0052	0.0513	-0.0296	0.0289	0.0280	0.0160	0.0020	-0.0030	-0.0174	-0.0095	-0.0069	-0.0028	-0.0022	-0.0057	-0.0004	0.0027	-0.0016	-0.0017	-0.0049	-0.0008	0.0004	0.0005	-0.0007	-0.0012	-0.0009	0.0008
L. platycalyx	2010	9	IMG0695	*Anthophora*	23.40	-0.1620	-0.0472	-0.0748	-0.0196	-0.0423	0.0074	-0.0277	-0.0448	0.0143	-0.0023	-0.0087	0.0228	-0.0057	-0.0006	0.0016	0.0031	0.0003	-0.0045	-0.0018	-0.0026	-0.0012	0.0033	0.0012	0.0013	0.0003	-0.0029	0.0005	-0.0001	-0.0005	0.0000
L. platycalyx	2010	10	IMG0702	*Anthophora*	22.23	-0.0350	0.0206	0.0512	-0.0101	0.0295	0.0203	-0.0149	0.0114	0.0096	0.0039	0.0025	0.0065	-0.0071	0.0032	0.0037	-0.0045	0.0004	-0.0023	0.0047	0.0017	0.0008	0.0013	0.0007	-0.0003	-0.0005	-0.0016	0.0003	0.0000	0.0003	0.0004
L. platycalyx	2010	11	IMG0705	*Anthophora*	19.70	0.0649	-0.0436	0.0111	-0.0462	-0.0298	-0.0135	-0.0217	-0.0297	0.0073	-0.0025	0.0069	-0.0039	-0.0007	-0.0001	0.0003	0.0020	-0.0030	-0.0018	0.0010	0.0003	-0.0011	0.0013	-0.0003	-0.0006	-0.0010	0.0001	0.0001	0.0003	0.0000	0.0001
L. platycalyx	2010	12	IMG0709	*Anthophora*	23.29	0.0381	-0.0207	-0.0131	-0.0522	-0.0244	0.0093	-0.0118	0.0036	-0.0134	0.0092	-0.0034	-0.0075	0.0050	0.0051	0.0017	-0.0017	0.0060	-0.0049	0.0025	-0.0026	0.0021	-0.0021	-0.0001	-0.0010	0.0011	0.0007	0.0004	-0.0001	-0.0003	-0.0002
L. platycalyx	2010	13	IMG0712	*Anthophora*	24.31	0.1742	-0.0053	-0.0107	-0.0258	-0.0094	0.0130	-0.0098	-0.0264	-0.0062	-0.0125	0.0174	0.0110	-0.0030	-0.0002	-0.0005	-0.0050	-0.0070	0.0017	-0.0019	-0.0010	-0.0008	0.0015	-0.0007	-0.0019	0.0003	0.0006	-0.0008	0.0005	0.0008	0.0002
L. platycalyx	2010	14	IMG0715	*Anthophora*	20.78	0.0483	-0.0692	0.0157	-0.0075	-0.0174	0.0060	0.0028	-0.0138	-0.0047	0.0118	-0.0009	-0.0050	0.0192	0.0016	-0.0030	-0.0078	0.0036	-0.0007	-0.0002	-0.0010	0.0010	0.0017	0.0016	-0.0010	0.0003	-0.0002	0.0001	-0.0002	-0.0001	0.0001
L. platycalyx	2010	15	IMG0720	*Anthophora*	22.59	-0.0814	-0.0090	-0.0179	-0.0879	0.0046	0.0302	0.0165	0.0054	0.0222	0.0117	-0.0155	0.0059	0.0028	0.0020	0.0049	0.0012	0.0005	-0.0010	-0.0035	-0.0002	0.0009	-0.0001	0.0008	-0.0004	-0.0012	-0.0006	-0.0003	0.0002	-0.0001	-0.0002
L. platycalyx	2010	16	IMG0723	*Anthophora*	21.17	-0.0204	-0.0361	0.0450	-0.0468	0.0064	-0.0017	-0.0103	-0.0057	0.0083	0.0166	0.0093	0.0057	0.0086	0.0014	0.0014	-0.0010	0.0003	0.0006	-0.0012	-0.0016	0.0009	-0.0008	0.0008	0.0003	0.0001	-0.0006	0.0000	0.0001	-0.0001	0.0000
L. platycalyx	2010	17	IMG0727	*Anthophora*	22.53	0.0203	-0.0095	-0.0085	-0.0672	-0.0075	0.0115	-0.0207	-0.0197	0.0097	0.0122	-0.0100	-0.0002	0.0087	-0.0049	-0.0002	-0.0018	0.0014	0.0036	0.0019	-0.0005	0.0009	0.0001	0.0003	-0.0002	-0.0008	0.0000	-0.0001	0.0000	-0.0001	0.0002
L. platycalyx	2010	18	IMG0628	*Anthophora*	22.07	-0.0537	0.0217	0.0131	-0.0190	-0.0440	-0.0208	0.0188	0.0157	0.0018	-0.0007	-0.0055	-0.0071	0.0088	0.0121	-0.0115	-0.0048	0.0080	-0.0009	0.0003	-0.0006	0.0011	-0.0015	0.0003	-0.0002	-0.0007	0.0005	-0.0003	-0.0003	0.0001	0.0001
L. platycalyx	2010	19	IMG0647	*Anthophora*	23.44	0.0414	0.0224	-0.0375	-0.0810	-0.0002	-0.0065	-0.0010	0.0059	0.0089	-0.0062	0.0035	0.0155	-0.0113	-0.0045	0.0084	-0.0015	-0.0052	-0.0017	-0.0027	-0.0001	0.0015	0.0007	-0.0009	-0.0005	0.0006	0.0001	0.0003	-0.0002	0.0000	0.0002
L. platycalyx	2010	20	IMG0652	*Anthophora*	23.28	0.0424	-0.0198	-0.0040	-0.0577	-0.0041	-0.0140	0.0050	0.0017	0.0141	-0.0079	-0.0024	0.0107	-0.0073	-0.0047	-0.0003	-0.0030	0.0002	0.0033	-0.0040	0.0009	0.0005	0.0001	0.0005	0.0007	-0.0003	0.0000	0.0000	0.0000	0.0001	0.0002
L. platycalyx	2010	21	IMG0657	*Anthophora*	24.61	0.0188	-0.0061	-0.0066	-0.0616	-0.0041	0.0095	0.0266	-0.0041	-0.0029	-0.0003	-0.0095	0.0056	-0.0040	-0.0039	0.0029	0.0038	-0.0021	-0.0013	-0.0032	0.0015	0.0000	0.0012	-0.0002	0.0003	-0.0006	0.0002	0.0001	0.0000	0.0001	0.0000
L. platycalyx	2010	22	IMG0663	*Anthophora*	24.59	0.0363	-0.0458	0.0022	-0.0600	-0.0259	-0.0363	0.0035	-0.0140	0.0024	-0.0262	0.0066	-0.0008	-0.0019	0.0098	0.0090	0.0019	-0.0029	0.0025	-0.0013	0.0014	0.0007	-0.0007	0.0007	0.0005	0.0001	0.0000	-0.0001	-0.0003	0.0001	0.0000
L. platycalyx	2010	23	IMG0668	*Anthophora*	25.15	-0.0416	0.0070	0.0091	-0.0718	0.0217	0.0242	0.0097	0.0038	0.0136	-0.0116	0.0113	0.0103	0.0057	0.0017	0.0033	0.0023	-0.0021	-0.0001	-0.0014	-0.0007	0.0000	-0.0010	0.0009	0.0000	-0.0006	-0.0002	-0.0001	0.0000	0.0000	0.0000
L. platycalyx	2012	24	IMG0994	*Anthophora*	20.26	0.0388	0.0340	0.0363	-0.0292	0.0242	0.0109	0.0039	-0.0019	-0.0050	-0.0117	0.0083	0.0135	-0.0057	0.0046	-0.0003	-0.0025	-0.0011	-0.0001	0.0010	0.0013	-0.0015	0.0022	-0.0002	0.0011	-0.0003	-0.0007	0.0000	0.0001	0.0000	0.0000
L. platycalyx	2012	25	IMG0998	*Anthophora*	17.33	0.0741	0.0129	0.0154	0.0110	0.0527	0.0064	0.0127	0.0291	0.0127	0.0006	-0.0077	0.0180	-0.0220	-0.0104	-0.0017	-0.0006	0.0004	-0.0040	-0.0008	0.0008	-0.0013	-0.0035	0.0003	0.0003	-0.0016	0.0012	0.0011	-0.0003	0.0003	-0.0004
L. platycalyx	2012	26	IMG1001	*Anthophora*	17.56	0.0848	-0.0291	0.0685	0.0016	0.0231	0.0216	-0.0302	0.0125	0.0048	-0.0115	0.0073	0.0143	-0.0081	-0.0006	-0.0025	-0.0074	0.0011	0.0000	0.0018	0.0013	0.0013	-0.0009	0.0012	-0.0004	-0.0003	-0.0002	-0.0003	-0.0005	-0.0002	-0.0002
L. platycalyx	2012	27	IMG1006	*Anthophora*	21.01	-0.0893	-0.0025	-0.0153	-0.0170	0.0287	-0.0117	0.0044	0.0402	0.0164	-0.0048	-0.0083	0.0077	0.0106	0.0029	-0.0064	0.0041	0.0021	0.0002	-0.0047	-0.0024	-0.0003	0.0017	-0.0027	0.0001	-0.0005	-0.0002	0.0008	0.0000	-0.0007	-0.0003
L. platycalyx	2012	28	IMG1009	*Anthophora*	18.83	0.1284	0.0105	0.0044	-0.0227	-0.0004	0.0126	0.0173	0.0077	-0.0146	-0.0217	-0.0043	0.0075	-0.0067	-0.0053	-0.0122	-0.0044	0.0017	0.0020	-0.0010	-0.0002	0.0021	-0.0014	0.0014	0.0017	-0.0010	0.0007	-0.0010	-0.0013	0.0002	-0.0004
L. platycalyx	2012	29	IMG1012	*Anthophora*	23.19	-0.0614	0.0346	0.0147	0.0145	-0.0065	0.0006	0.0050	0.0180	0.0211	0.0029	-0.0183	0.0049	0.0008	0.0060	0.0024	-0.0039	0.0012	-0.0009	-0.0013	0.0001	0.0007	0.0002	0.0001	0.0002	-0.0005	-0.0005	-0.0001	0.0001	-0.0003	0.0000
L. platycalyx	2012	30	IMG1015b	*Anthophora*	18.98	0.0640	-0.0201	0.0398	0.0000	0.0074	-0.0138	0.0066	0.0147	-0.0054	-0.0104	-0.0106	0.0141	-0.0060	0.0176	-0.0017	0.0032	-0.0004	-0.0033	-0.0008	0.0034	-0.0017	0.0007	0.0006	0.0030	0.0016	-0.0002	-0.0005	0.0002	-0.0002	-0.0001
L. platycalyx	2012	31	IMG1018b	*Anthophora*	18.70	0.0280	0.0307	0.0664	0.0073	-0.0014	0.0267	0.0307	0.0086	0.0123	-0.0191	0.0014	0.0130	0.0047	0.0040	-0.0068	-0.0038	0.0015	0.0010	-0.0009	-0.0012	0.0005	-0.0010	0.0009	-0.0008	-0.0011	0.0004	-0.0003	0.0004	-0.0003	0.0004
L. platycalyx	2012	32	IMG1021b	*Anthophora*	17.77	0.1288	-0.0557	0.0142	0.0190	0.0051	0.0060	0.0189	-0.0018	0.0133	-0.0142	-0.0094	0.0174	-0.0034	-0.0009	0.0053	-0.0066	-0.0024	0.0016	-0.0008	0.0038	0.0038	-0.0008	0.0023	-0.0003	0.0016	-0.0007	-0.0004	-0.0002	-0.0002	0.0001
L. platycalyx	2012	33	IMG1030	*Anthophora*	18.94	0.0242	-0.0135	0.0786	-0.0144	-0.0190	0.0057	-0.0137	0.0230	0.0191	-0.0025	0.0185	0.0128	-0.0110	0.0010	-0.0014	-0.0012	0.0010	0.0022	0.0038	-0.0003	-0.0001	-0.0019	-0.0025	-0.0011	0.0000	-0.0006	-0.0007	-0.0005	-0.0001	0.0007
L. polygalyfolia	2010	1	IMG1301	*Xylocopa*	30.71	0.0334	0.0983	-0.0242	-0.0309	-0.0213	-0.0179	-0.0054	0.0071	-0.0013	0.0099	-0.0148	-0.0014	-0.0086	0.0039	-0.0046	-0.0095	-0.0001	0.0028	-0.0004	-0.0008	0.0011	-0.0025	0.0002	0.0012	0.0016	0.0004	0.0004	-0.0006	0.0005	-0.0002
L. polygalyfolia	2010	2	IMG1313	*Xylocopa*	30.22	0.0208	0.0864	-0.0161	-0.0278	-0.0171	0.0081	0.0118	-0.0020	-0.0057	0.0112	-0.0152	-0.0023	-0.0038	0.0073	0.0011	-0.0035	0.0013	0.0000	0.0006	-0.0001	0.0015	-0.0013	-0.0006	-0.0003	0.0003	0.0003	0.0004	0.0000	0.0003	-0.0001
L. polygalyfolia	2010	3	IMG1319	*Xylocopa*	30.76	-0.0158	0.1155	-0.0528	-0.0183	-0.0134	-0.0020	0.0045	-0.0024	0.0093	-0.0010	-0.0065	0.0018	0.0018	-0.0021	-0.0017	0.0022	-0.0002	-0.0076	-0.0024	0.0001	-0.0005	0.0001	0.0001	-0.0007	0.0009	-0.0006	-0.0003	0.0001	-0.0003	-0.0001
L. polygalyfolia	2010	4	IMG1322	*Xylocopa*	30.97	-0.0147	0.0734	-0.0198	0.0041	-0.0241	0.0042	0.0066	0.0012	-0.0143	0.0022	-0.0081	-0.0012	-0.0001	-0.0048	0.0009	-0.0056	0.0005	-0.0025	-0.0015	0.0005	0.0019	0.0002	-0.0009	-0.0006	0.0007	0.0001	-0.0001	0.0000	0.0000	-0.0001
L. polygalyfolia	2010	5	IMG1325	*Xylocopa*	31.66	0.0156	0.1078	-0.0346	-0.0309	-0.0214	-0.0125	-0.0089	0.0045	-0.0089	-0.0021	-0.0140	-0.0017	-0.0115	-0.0023	0.0038	-0.0024	-0.0005	0.0015	0.0007	-0.0028	0.0043	-0.0005	-0.0015	-0.0003	0.0025	0.0000	-0.0001	-0.0001	-0.0003	-0.0002
L. polygalyfolia	2010	6	IMG1328	*Xylocopa*	30.98	0.0307	0.0923	-0.0304	0.0018	-0.0116	0.0145	0.0165	0.0051	-0.0055	0.0018	-0.0097	0.0023	-0.0015	-0.0028	0.0044	-0.0081	-0.0001	0.0035	-0.0017	-0.0006	0.0023	-0.0003	-0.0008	-0.0005	0.0008	0.0005	0.0000	-0.0003	0.0002	0.0000

L. polygalyfolia	2010	7	IMG1331	*Xylocopa*	30.50	-0.0006	0.0818	-0.0355	-0.0253	0.0005	0.0155	0.0144	0.0083	0.0000	-0.0041	-0.0122	0.0019	0.0032	-0.0019	0.0047	0.0024	-0.0045	0.0010	0.0010	0.0006	0.0005	0.0000	-0.0001	-0.0008	-0.0002	0.0006	-0.0001	0.0001	0.0000	0.0001
L. polygalyfolia	2010	8	IMG1334	*Xylocopa*	31.07	-0.0502	0.0639	-0.0685	-0.0056	-0.0248	-0.0045	0.0039	-0.0011	0.0031	-0.0073	-0.0238	0.0014	-0.0064	-0.0010	0.0030	-0.0063	-0.0029	-0.0018	-0.0010	0.0011	0.0005	-0.0007	0.0002	-0.0001	0.0002	-0.0006	-0.0003	-0.0003	0.0000	-0.0001
L. polygalyfolia	2010	9	IMG1337	*Xylocopa*	32.42	-0.0953	0.0862	-0.0918	-0.0086	-0.0281	-0.0079	-0.0085	-0.0045	0.0040	-0.0128	-0.0141	0.0026	-0.0045	0.0019	0.0007	-0.0017	-0.0032	-0.0028	0.0000	-0.0018	-0.0026	0.0004	0.0000	-0.0010	-0.0001	-0.0011	-0.0006	-0.0005	-0.0003	-0.0002
L. polygalyfolia	2010	10	IMG1340	*Xylocopa*	28.38	0.0123	0.0877	-0.0119	0.0118	0.0076	-0.0088	-0.0095	-0.0104	0.0048	0.0194	-0.0208	0.0003	-0.0011	-0.0024	-0.0014	0.0028	0.0046	0.0007	-0.0026	-0.0013	0.0018	-0.0011	0.0002	0.0001	-0.0002	-0.0004	-0.0002	-0.0002	-0.0001	0.0002
L. polygalyfolia	2010	11	IMG1346	*Xylocopa*	27.33	-0.0295	0.0945	-0.0755	0.0026	-0.0089	0.0113	-0.0097	0.0046	-0.0017	0.0023	-0.0170	0.0008	0.0040	0.0039	0.0047	0.0004	-0.0021	-0.0026	-0.0002	0.0005	0.0004	-0.0006	-0.0010	0.0001	-0.0001	0.0001	0.0001	-0.0002	0.0003	-0.0003
L. polygalyfolia	2010	12	IMG1350	*Xylocopa*	27.17	0.0334	0.1025	-0.0176	-0.0005	0.0240	0.0154	0.0142	0.0046	-0.0034	0.0137	-0.0169	0.0077	-0.0027	0.0078	-0.0027	0.0016	0.0019	-0.0012	-0.0001	0.0004	0.0002	0.0004	0.0004	0.0004	0.0002	-0.0003	0.0000	-0.0001	0.0000	-0.0002
L. polygalyfolia	2010	13	IMG1353	*Xylocopa*	31.81	-0.0383	0.0703	-0.0343	0.0068	0.0092	-0.0065	0.0111	0.0034	0.0061	-0.0012	-0.0066	0.0017	0.0078	0.0040	-0.0036	-0.0035	-0.0013	0.0012	0.0069	0.0010	0.0009	-0.0009	-0.0003	0.0004	-0.0007	0.0010	0.0005	0.0000	0.0006	0.0004
L. polygalyfolia	2010	14	IMG1356	*Xylocopa*	32.19	0.0597	0.0876	-0.0074	0.0009	0.0110	-0.0001	0.0344	-0.0028	0.0040	0.0017	-0.0063	0.0082	0.0059	0.0050	-0.0077	-0.0050	-0.0050	0.0042	0.0031	0.0008	-0.0010	-0.0006	-0.0004	0.0008	0.0002	0.0005	-0.0002	0.0002	0.0001	0.0003
L. polygalyfolia	2010	15	IMG1359	*Xylocopa*	32.25	-0.0101	0.0903	-0.0437	0.0093	0.0057	-0.0050	0.0166	-0.0006	0.0131	-0.0022	-0.0088	0.0042	0.0044	0.0007	-0.0041	-0.0027	-0.0035	0.0002	0.0002	0.0015	0.0006	-0.0001	-0.0004	0.0004	-0.0001	0.0005	0.0005	-0.0002	0.0001	0.0001
L. polygalyfolia	2010	16	IMG1362	*Xylocopa*	33.83	-0.0032	0.1093	-0.0233	-0.0273	-0.0121	-0.0028	0.0343	0.0015	0.0116	-0.0108	0.0044	0.0048	-0.0045	-0.0001	-0.0046	-0.0003	0.0039	-0.0025	-0.0022	0.0001	-0.0005	0.0029	0.0001	-0.0009	0.0012	-0.0021	0.0003	0.0000	0.0000	-0.0001
L. polygalyfolia	2010	17	IMG1366	*Xylocopa*	33.43	0.0087	0.1102	-0.0407	0.0125	0.0020	-0.0284	0.0283	-0.0010	0.0060	0.0034	-0.0045	0.0081	-0.0019	-0.0033	-0.0039	-0.0041	0.0018	0.0006	0.0008	0.0028	0.0019	0.0000	0.0001	0.0002	0.0003	-0.0002	0.0004	0.0001	0.0002	0.0000
L. polygalyfolia	2010	18	IMG1370	*Xylocopa*	33.18	0.0119	0.0993	-0.0563	-0.0141	-0.0127	-0.0196	0.0155	-0.0109	-0.0034	-0.0068	-0.0013	0.0012	0.0050	-0.0034	-0.0083	-0.0016	-0.0014	0.0000	-0.0005	0.0029	0.0010	0.0008	-0.0002	0.0005	0.0005	0.0006	0.0006	-0.0002	0.0000	0.0000
L. polygalyfolia	2010	19	IMG1374	*Xylocopa*	34.19	-0.0080	0.1189	-0.0146	-0.0046	-0.0188	-0.0226	0.0320	-0.0071	0.0049	0.0002	0.0041	-0.0020	-0.0005	0.0016	-0.0008	0.0031	-0.0029	-0.0022	-0.0007	0.0016	-0.0003	-0.0004	-0.0001	-0.0007	-0.0001	-0.0003	0.0002	0.0000	0.0001	0.0000
L. polygalyfolia	2010	20	IMG1378	*Xylocopa*	33.40	-0.0168	0.0959	-0.0533	-0.0053	0.0047	-0.0097	0.0247	0.0072	0.0254	0.0079	-0.0077	0.0073	0.0042	-0.0006	-0.0057	0.0037	0.0050	0.0038	0.0006	-0.0039	0.0007	-0.0003	-0.0009	-0.0002	0.0006	-0.0001	-0.0002	0.0001	-0.0002	0.0000
L. polygalyfolia	2013	21	IMG2806	*Xylocopa*	31.80	0.0174	0.0854	0.0049	-0.0300	-0.0076	-0.0104	0.0249	-0.0128	-0.0022	0.0059	0.0040	0.0035	0.0090	0.0037	-0.0053	-0.0005	0.0028	0.0016	0.0023	-0.0006	0.0001	-0.0020	-0.0002	-0.0009	-0.0001	0.0001	0.0003	0.0000	0.0002	0.0001
L. polygalyfolia	2013	22	IMG2809	*Xylocopa*	26.52	0.1383	0.0618	0.0047	-0.0179	0.0065	0.0257	0.0027	-0.0097	-0.0241	0.0236	-0.0131	0.0038	0.0050	-0.0023	-0.0099	0.0043	-0.0025	0.0014	0.0001	-0.0031	-0.0046	-0.0009	0.0006	-0.0016	0.0001	0.0006	-0.0012	0.0006	0.0001	0.0002
L. polygalyfolia	2013	23	IMG2812	*Xylocopa*	27.36	0.1106	0.0928	0.0155	-0.0122	-0.0069	0.0306	0.0145	-0.0232	-0.0133	-0.0046	0.0015	0.0053	0.0024	-0.0050	-0.0074	-0.0030	-0.0062	0.0017	-0.0005	0.0017	-0.0023	0.0032	-0.0011	0.0026	-0.0007	0.0019	-0.0014	0.0008	0.0006	0.0005
L. polygalyfolia	2013	24	IMG2820	*Xylocopa*	27.87	0.0642	0.0381	-0.0155	-0.0166	0.0085	0.0039	0.0015	-0.0192	-0.0066	0.0068	-0.0059	-0.0032	0.0096	0.0031	-0.0038	0.0008	0.0002	0.0053	0.0049	-0.0011	-0.0016	0.0007	0.0002	-0.0002	-0.0001	-0.0009	-0.0001	0.0001	0.0000	0.0000
L. polygalyfolia	2013	25	IMG2811	*Xylocopa*	27.32	0.0514	0.0851	0.0054	-0.0038	0.0159	0.0219	0.0024	-0.0052	-0.0026	0.0048	-0.0095	0.0045	0.0004	-0.0086	-0.0077	-0.0079	0.0015	0.0027	-0.0013	-0.0012	0.0018	0.0007	0.0008	0.0001	-0.0003	0.0003	-0.0001	-0.0007	0.0001	0.0000
L. polygalyfolia	2013	26	IMG2815	*Xylocopa*	25.16	0.0906	0.0812	-0.0133	0.0288	0.0133	0.0312	0.0040	0.0004	0.0136	-0.0116	-0.0033	0.0081	0.0001	0.0028	-0.0035	0.0020	-0.0008	0.0008	0.0006	0.0007	-0.0005	-0.0002	0.0001	0.0006	0.0001	-0.0002	-0.0002	0.0002	0.0001	-0.0001
L. tristis	2010	1	IMG0902	Megachilidae	26.96	-0.0790	-0.0135	-0.0100	-0.0330	0.0534	-0.0052	0.0017	-0.0246	0.0048	0.0047	-0.0018	-0.0225	-0.0114	-0.0063	0.0027	0.0042	0.0026	-0.0050	0.0022	0.0096	-0.0032	-0.0021	-0.0001	-0.0003	0.0006	-0.0025	-0.0002	-0.0006	0.0006	0.0003
L. tristis	2010	2	IMG0912	Megachilidae	26.93	-0.0906	-0.0470	0.0069	0.0306	0.0105	0.0157	0.0047	-0.0420	0.0225	0.0088	0.0031	-0.0152	-0.0043	-0.0020	-0.0054	0.0021	-0.0068	0.0012	-0.0019	-0.0043	0.0017	-0.0006	-0.0001	-0.0006	-0.0005	0.0004	-0.0005	0.0002	0.0002	0.0000
L. tristis	2010	3	IMG0915	Megachilidae	23.61	-0.0270	-0.0505	0.0197	-0.0088	0.0402	0.0048	0.0177	-0.0182	0.0106	-0.0031	-0.0025	-0.0120	0.0042	-0.0123	-0.0009	-0.0055	0.0086	-0.0045	0.0016	-0.0008	0.0005	-0.0006	-0.0017	0.0003	0.0010	0.0000	0.0019	-0.0002	-0.0004	-0.0001
L. tristis	2010	4	IMG0926	Megachilidae	26.27	-0.0186	-0.0681	0.0123	-0.0127	0.0123	0.0232	0.0386	-0.0326	-0.0112	0.0068	0.0054	-0.0136	0.0108	-0.0025	0.0031	0.0023	-0.0025	-0.0031	-0.0004	0.0030	-0.0030	0.0007	0.0000	0.0012	0.0000	0.0003	-0.0002	-0.0009	-0.0006	0.0002
L. tristis	2010	5	IMG0934	Megachilidae	24.31	-0.1078	-0.0236	0.0375	0.0203	0.0153	0.0049	-0.0221	-0.0329	0.0124	-0.0048	0.0035	-0.0106	-0.0180	0.0035	-0.0035	0.0023	-0.0040	0.0064	-0.0073	0.0033	-0.0006	-0.0021	0.0020	-0.0025	0.0018	0.0000	-0.0009	-0.0003	-0.0002	-0.0012
L. tristis	2010	6	IMG0938	Megachilidae	23.06	-0.0783	-0.0748	0.0384	-0.0063	0.0226	0.0018	0.0224	-0.0254	0.0127	-0.0011	0.0045	-0.0168	-0.0147	-0.0011	0.0018	0.0041	0.0008	-0.0029	-0.0020	0.0042	-0.0027	-0.0009	0.0012	-0.0007	0.0003	0.0002	-0.0010	-0.0003	0.0010	-0.0003
L. tristis	2010	7	IMG0946	Megachilidae	25.10	-0.0451	-0.0703	0.0046	0.0132	0.0309	-0.0136	-0.0045	-0.0396	-0.0016	0.0198	-0.0098	-0.0088	-0.0088	-0.0039	-0.0100	-0.0015	-0.0051	0.0042	-0.0066	-0.0021	0.0002	-0.0012	-0.0030	-0.0013	-0.0006	0.0002	0.0001	0.0003	-0.0004	0.0002
L. tristis	2010	8	IMG0953	Megachilidae	24.41	-0.0718	-0.0569	0.0318	0.0179	0.0249	0.0112	-0.0073	-0.0406	0.0199	0.0039	0.0062	-0.0187	0.0012	0.0041	0.0088	-0.0032	0.0036	0.0066	0.0001	0.0053	-0.0019	-0.0029	-0.0011	-0.0003	0.0008	0.0028	-0.0020	-0.0005	-0.0001	-0.0008
L. tristis	2010	9	IMG0965	Megachilidae	21.93	-0.0324	-0.0563	0.0315	0.0063	0.0410	0.0031	0.0050	-0.0295	0.0164	0.0001	-0.0085	-0.0109	-0.0070	0.0035	-0.0120	-0.0010	0.0060	0.0055	-0.0048	-0.0064	0.0034	-0.0001	-0.0041	0.0002	0.0018	0.0007	-0.0002	0.0003	-0.0001	-0.0006
L. tristis	2010	10	IMG0968	Megachilidae	24.13	-0.0899	-0.0286	-0.0241	-0.0039	0.0293	-0.0117	-0.0056	-0.0193	0.0149	0.0231	-0.0010	0.0030	-0.0127	-0.0029	-0.0111	0.0106	-0.0041	0.0028	0.0020	0.0026	0.0053	0.0037	0.0018	-0.0018	0.0018	-0.0003	0.0014	0.0003	0.0010	0.0009
L. tristis	2010	11	IMG0971	Megachilidae	22.54	-0.0682	-0.0546	0.0041	-0.0075	0.0167	-0.0042	-0.0109	-0.0215	0.0191	-0.0011	-0.0178	-0.0098	0.0033	-0.0084	0.0127	-0.0073	0.0150	0.0149	-0.0042	0.0120	-0.0082	-0.0006	-0.0027	-0.0004	0.0018	0.0002	-0.0003	0.0009	-0.0005	-0.0002
L. tristis	2010	12	IMG0976	Megachilidae	23.55	-0.0044	-0.0363	0.0148	0.0174	0.0413	0.0131	0.0260	-0.0190	0.0036	-0.0022	-0.0034	-0.0096	0.0059	0.0041	0.0004	-0.0075	0.0056	-0.0089	0.0055	-0.0007	0.0030	0.0008	0.0002	-0.0005	0.0010	-0.0008	0.0013	0.0000	-0.0010	-0.0001
L. tristis	2010	13	IMG0983	Megachilidae	24.08	-0.0240	-0.0362	0.0164	-0.0034	0.0287	0.0006	0.0182	-0.0286	-0.0051	0.0005	-0.0061	-0.0103	0.0120	-0.0048	0.0023	0.0002	0.0020	-0.0045	0.0035	0.0018	0.0008	0.0006	-0.0011	0.0007	0.0005	0.0000	0.0003	-0.0006	-0.0004	0.0001
L. tristis	2010	14	IMG0986	Megachilidae	24.62	-0.0366	-0.0330	0.0046	0.0122	0.0453	-0.0122	0.0312	-0.0204	0.0029	0.0115	-0.0011	-0.0133	0.0041	-0.0003	-0.0027	0.0006	0.0008	-0.0044	0.0023	-0.0027	0.0017	-0.0008	-0.0025	-0.0005	0.0010	-0.0008	0.0001	0.0002	0.0000	0.0001
L. tristis	2010	15	IMG0989	Megachilidae	24.31	-0.0128	-0.0456	0.0009	0.0042	0.0402	-0.0023	0.0048	-0.0310	0.0101	-0.0042	-0.0046	-0.0116	-0.0015	0.0019	-0.0027	-0.0032	0.0014	0.0036	0.0031	-0.0007	0.0010	0.0003	-0.0022	0.0003	0.0006	0.0003	0.0002	0.0005	-0.0003	0.0002
L. tristis	2010	16	IMG0993	Megachilidae	22.64	0.0492	-0.0056	0.0240	0.0251	0.0236	0.0072	0.0305	-0.0369	0.0141	-0.0071	-0.0036	-0.0011	0.0036	0.0085	0.0053	0.0103	0.0041	0.0051	0.0034	0.0013	-0.0057	-0.0016	-0.0035	-0.0008	-0.0004	-0.0002	-0.0004	-0.0010	0.0000	0.0007
L. tristis	2010	17	IMG0996	Megachilidae	24.25	-0.0426	-0.0638	0.0198	0.0024	0.0508	0.0010	0.0143	-0.0181	0.0003	-0.0071	-0.0032	-0.0070	0.0017	0.0028	-0.0024	-0.0025	-0.0016	-0.0045	0.0005	-0.0020	0.0021	0.0005	-0.0008	0.0009	0.0016	-0.0002	0.0003	0.0000	-0.0001	0.0000
L. tristis	2010	18	IMG1004	Megachilidae	24.20	-0.0150	-0.0496	0.0192	0.0281	0.0308	0.0141	0.0260	-0.0117	0.0246	-0.0083	0.0001	-0.0034	0.0088	0.0057	-0.0002	0.0006	0.0000	0.0053	0.0001	-0.0027	0.0037	-0.0001	-0.0016	0.0000	0.0002	0.0004	-0.0005	0.0002	-0.0001	-0.0003
L. tristis	2010	19	IMG1008	Megachilidae	24.01	-0.0949	-0.0298	-0.0078	-0.0218	0.0472	0.0213	-0.0091	-0.0282	-0.0039	-0.0173	-0.0009	0.0047	-0.0029	-0.0139	-0.0029	-0.0045	-0.0120	0.0076	0.0000	0.0027	-0.0008	-0.0014	0.0010	0.0008	0.0005	-0.0006	0.0007	-0.0012	-0.0002	0.0003
L. tristis	2010	20	IMG1018	Megachilidae	24.06	-0.0569	-0.0486	0.0293	-0.0141	0.0459	0.0040	-0.0110	-0.0078	0.0167	-0.0131	-0.0017	-0.0003	0.0015	0.0016	-0.0008	-0.0005	-0.0024	0.0025	0.0030	-0.0026	0.0035	0.0002	-0.0004	0.0011	0.0002	0.0001	-0.0001	0.0001	0.0000	0.0002
L. tristis	2010	21	IMG0931	Megachilidae	22.86	-0.0774	-0.0798	-0.0124	-0.0070	0.0312	-0.0138	-0.0136	-0.0007	0.0030	-0.0154	-0.0027	0.0127	0.0069	-0.0201	-0.0065	-0.0017	-0.0075	-0.0015	-0.0024	-0.0037	0.0007	-0.0014	-0.0029	0.0017	-0.0005	-0.0005	0.0002	-0.0006	0.0002	0.0001
L. tristis	2010	22	IMG0942	Megachilidae	23.72	-0.0837	-0.0506	0.0083	-0.0264	0.0290	-0.0353	0.0019	-0.0004	0.0206	-0.0109	-0.0152	-0.0031	-0.0180	-0.0031	0.0007	-0.0113	0.0025	-0.0039	0.0014	-0.0044	0.0007	-0.0019	0.0025	0.0003	-0.0009	0.0012	-0.0001	0.0016	0.0001	0.0006
L. tristis	2010	23	IMG0950	Megachilidae	25.25	-0.1434	-0.0148	0.0064	0.0087	0.0086	-0.0003	-0.0016	-0.0159	0.0068	-0.0144	0.0116	-0.0007	-0.0111	0.0013	-0.0124	0.0012	-0.0027	-0.0108	0.0007	-0.0010	0.0026	0.0002	0.0016	-0.0006	0.0030	-0.0003	-0.0007	0.0001	0.0019	0.0008
L. tristis	2010	24	IMG0958	Megachilidae	24.90	-0.0256	-0.0678	-0.0048	0.0074	-0.0008	-0.0206	-0.0159	-0.0218	-0.0146	-0.0195	-0.0060	-0.0031	0.0112	-0.0036	0.0043	-0.0023	-0.0103	0.0015	-0.0014	-0.0018	0.0016	-0.0012	0.0008	0.0012	-0.0015	-0.0015	-0.0002	-0.0012	0.0003	-0.0002
L. tristis	2010	25	IMG0961	Megachilidae	22.69	-0.0085	-0.0306	0.0172	0.0188	0.0241	0.0221	0.0125	-0.0278	0.0177	-0.0244	-0.0044	-0.0020	0.0049	0.0069	0.0037	0.0022	0.0001	-0.0090	0.0037	-0.0038	0.0055	0.0009	0.0015	0.0003	0.0001	0.0016	0.0001	-0.0003	-0.0002	-0.0002
L. tristis	2010	26	IMG0981	Megachilidae	25.22	-0.0046	-0.0132	0.0518	-0.0132	0.0041	-0.0394	0.0177	0.0110	-0.0023	-0.0043	-0.0075	-0.0079	-0.0045	-0.0123	-0.0083	0.0030	0.0031	0.0016	0.0089	0.0037	0.0002	0.0006	0.0000	0.0007	-0.0012	-0.0027	0.0013	0.0004	-0.0001	-0.0009
L. tristis	2010	27	IMG1000	Megachilidae	24.17	-0.0471	-0.0533	0.0461	0.0285	0.0303	0.0050	0.0185	-0.0063	0.0065	-0.0203	0.0125	-0.0028	0.0098	-0.0001	-0.0027	-0.0020	-0.0084	-0.0030	0.0031	-0.0029	0.0035	-0.0005	0.0001	0.0015	-0.0003	0.0006	0.0000	-0.0005	-0.0003	0.0002
L. tristis	2010	28	IMG1015	Megachilidae	25.85	-0.0680	0.0272	0.0376	-0.0596	0.0301	-0.0258	-0.0221	0.0050	-0.0120	-0.0018	-0.0068	-0.0003	0.0021	0.0010	-0.0068	0.0058	0.0011	0.0008	0.0003	-0.0008	-0.0003	0.0018	-0.0002	-0.0003	-0.0003	0.0000	0.0004	0.0000	0.0003	0.0000
L. tristis	2010	29	IMG1021	Megachilidae	24.93	-0.0586	0.0271	0.0369	-0.0205	0.0281	-0.0175	0.0206	-0.0073	-0.0128	-0.0297	-0.0033	-0.0071	0.0042	0.0099	-0.0027	0.0025	-0.0001	-0.0039	0.0000	-0.0012	0.0004	0.0002	-0.0002	-0.0013	-0.0001	0.0009	0.0000	0.0000	0.0001	-0.0002
L. tristis	2012	30	IMG0965B	Megachilidae	23.16	-0.1275	-0.0140	-0.0158	-0.0016	0.0494	-0.0210	0.0050	-0.0073	-0.0453	-0.0105	-0.0102	0.0140	0.0068	-0.0052	-0.0028	-0.0028	-0.0021	0.0050	0.0047	-0.0011	0.0006	0.0014	-0.0014	-0.0020	0.0011	-0.0001	-0.0006	0.0005	0.0002	0.0001
L. tristis	2012	31	IMG0960	Megachilidae	21.76	-0.0670	0.0330	0.0288	0.0240	0.0059	-0.0321	0.0155	0.0040	-0.0218	-0.0021	0.0042	0.0094	0.0094	-0.0110	0.0031	0.0053	0.0117	-0.0031	-0.0061	0.0057	0.0024	0.0020	-0.0021	-0.0020	-0.0005	-0.0012	-0.0013	-0.0003	0.0000	0.0004
L. tristis	2012	32	IMG0956	Megachilidae	24.49	-0.2099	-0.0421	-0.0361	0.0195	0.0170	-0.0129	0.0162	0.0029	-0.0198	-0.0093	0.0345	0.0147	0.0000	0.0030	-0.0097	0.0084	0.0028	0.0018	-0.0007	-0.0005	0.0014	-0.0016	0.0020	-0.0003	0.0027	0.0013	0.0009	0.0016	0.0006	0.0006

L. tristis	2012	33	IMG0952	Megachilidae	22.82	-0.0112	-0.0495	0.0183	-0.0018	0.0433	-0.0393	0.0078	0.0083	-0.0050	-0.0093	0.0048	0.0050	0.0098	-0.0017	-0.0013	-0.0063	-0.0060	0.0065	-0.0009	-0.0017	0.0010	0.0007	-0.0006	0.0009	0.0014	-0.0006	0.0003	0.0001	-0.0003	0.0001
L. tristis	2012	34	IMG0946B	Megachilidae	24.61	-0.0843	0.0070	0.0149	0.0170	0.0455	-0.0108	0.0250	0.0027	0.0047	-0.0144	0.0117	-0.0031	0.0002	-0.0003	0.0047	-0.0039	-0.0005	-0.0002	-0.0047	-0.0035	-0.0001	-0.0022	0.0014	0.0004	-0.0006	0.0007	-0.0003	0.0005	0.0001	-0.0006
L. tristis	2012	35	IMG0940	Megachilidae	24.41	-0.0523	0.0472	0.0358	0.0423	0.0050	-0.0161	-0.0215	-0.0038	-0.0323	-0.0068	-0.0031	-0.0028	0.0056	-0.0010	-0.0041	0.0053	0.0115	-0.0006	-0.0028	0.0022	0.0045	0.0010	-0.0010	-0.0014	-0.0010	-0.0003	-0.0015	-0.0006	-0.0005	0.0010
L. tristis	2012	36	IMG0929	Megachilidae	22.84	-0.0335	-0.0232	0.0287	-0.0027	0.0266	0.0057	-0.0370	-0.0097	-0.0093	-0.0622	-0.0124	-0.0062	0.0009	0.0121	0.0097	-0.0029	0.0141	-0.0051	-0.0020	0.0008	-0.0025	0.0042	-0.0002	-0.0010	-0.0004	0.0034	0.0000	-0.0010	0.0004	0.0004
L. verticillata	2010	1	IMG2280	*Anthophora*	20.14	0.0775	0.0085	-0.0573	0.0238	-0.0069	0.0136	-0.0250	0.0099	0.0257	-0.0028	0.0010	-0.0037	0.0162	-0.0039	-0.0076	0.0047	-0.0021	-0.0006	0.0003	0.0031	-0.0024	-0.0012	-0.0009	-0.0011	0.0008	0.0003	-0.0001	0.0005	0.0003	-0.0003
L. verticillata	2010	2	IMG2286	*Anthophora*	21.00	0.0726	0.0190	-0.0065	0.0257	-0.0299	0.0212	-0.0075	0.0027	0.0238	0.0090	0.0021	0.0021	0.0021	0.0034	-0.0012	0.0027	-0.0008	-0.0018	0.0059	0.0021	-0.0013	0.0007	-0.0012	-0.0012	0.0003	0.0011	-0.0002	0.0004	0.0002	-0.0002
L. verticillata	2010	3	IMG2292	*Anthophora*	22.56	-0.0098	0.1070	0.0494	0.0413	-0.0287	0.0236	-0.0133	-0.0038	0.0212	0.0085	0.0219	-0.0067	0.0007	0.0098	-0.0069	-0.0020	0.0020	0.0020	0.0020	-0.0050	-0.0008	0.0003	0.0001	0.0007	0.0000	-0.0003	-0.0002	-0.0003	-0.0002	0.0000
L. verticillata	2010	4	IMG2299	*Anthophora*	21.59	0.0265	-0.0153	-0.0732	0.0399	0.0219	0.0066	-0.0459	-0.0174	0.0238	-0.0097	-0.0092	0.0030	0.0062	-0.0067	-0.0009	0.0009	0.0028	-0.0050	0.0002	-0.0023	0.0021	0.0011	-0.0015	0.0018	-0.0002	-0.0005	-0.0009	0.0007	0.0000	-0.0003
L. verticillata	2010	5	IMG2302	*Anthophora*	23.82	0.0238	0.0310	-0.0243	0.0186	0.0136	-0.0238	-0.0274	-0.0068	-0.0014	0.0183	0.0048	-0.0008	0.0125	0.0066	0.0064	0.0022	0.0005	0.0031	0.0041	-0.0044	-0.0010	0.0003	0.0006	0.0005	0.0003	-0.0004	0.0000	0.0001	0.0004	0.0000
L. verticillata	2010	6	IMG2305	*Anthophora*	25.03	0.0247	0.0888	-0.0218	-0.0328	0.0000	-0.0139	0.0030	-0.0151	-0.0026	0.0020	0.0182	0.0007	0.0099	0.0010	0.0004	0.0032	-0.0009	0.0013	-0.0002	-0.0008	-0.0011	-0.0025	-0.0002	0.0001	0.0002	0.0003	0.0004	-0.0002	0.0002	0.0001
L. verticillata	2010	7	IMG2309	*Anthophora*	21.52	0.0624	0.0412	-0.0018	-0.0300	-0.0166	-0.0360	-0.0376	-0.0140	-0.0027	-0.0197	0.0229	-0.0067	0.0036	-0.0007	-0.0001	-0.0048	0.0032	0.0014	0.0026	0.0000	0.0000	-0.0015	0.0006	0.0001	0.0008	-0.0003	0.0005	0.0000	-0.0001	0.0000
L. verticillata	2010	8	IMG2313	*Anthophora*	23.80	-0.0015	0.0539	-0.0224	0.0027	0.0198	0.0046	-0.0147	0.0052	0.0185	-0.0027	0.0086	0.0057	0.0132	-0.0034	0.0039	0.0000	-0.0037	0.0026	0.0041	-0.0004	0.0010	-0.0018	0.0002	-0.0002	-0.0004	-0.0005	-0.0002	-0.0002	0.0002	0.0001
L. verticillata	2010	9	IMG2316	*Anthophora*	24.37	-0.0362	0.1083	-0.0321	0.0148	-0.0010	-0.0163	-0.0180	-0.0101	0.0138	0.0096	0.0126	-0.0012	0.0060	0.0042	0.0044	0.0094	0.0025	0.0024	0.0005	-0.0025	0.0007	-0.0003	0.0003	0.0008	-0.0003	0.0009	-0.0004	-0.0004	-0.0002	-0.0002
L. verticillata	2010	10	IMG2322	*Anthophora*	19.69	0.0995	0.0548	-0.0260	0.0402	0.0301	0.0056	-0.0159	-0.0022	0.0258	-0.0081	0.0096	0.0120	-0.0021	-0.0009	-0.0029	0.0046	-0.0032	-0.0071	0.0005	0.0016	-0.0010	-0.0015	-0.0004	-0.0011	0.0004	0.0000	-0.0001	0.0000	-0.0003	-0.0005
L. verticillata	2010	11	IMG2325	*Anthophora*	22.57	0.0145	0.0710	-0.0410	0.0142	0.0156	-0.0285	-0.0132	-0.0115	0.0140	-0.0049	0.0102	0.0080	0.0004	-0.0057	0.0076	-0.0029	-0.0046	-0.0011	0.0014	0.0010	0.0004	-0.0006	0.0009	0.0001	-0.0009	-0.0001	-0.0001	-0.0003	0.0003	-0.0001
L. verticillata	2010	12	IMG2473	*Anthophora*	27.55	0.0188	0.0683	0.0113	0.0210	-0.0228	0.0015	-0.0087	-0.0062	-0.0013	0.0113	0.0145	-0.0062	0.0046	0.0007	-0.0004	-0.0071	0.0054	-0.0003	0.0026	-0.0028	0.0006	-0.0016	-0.0002	-0.0007	-0.0005	-0.0002	0.0001	0.0000	0.0003	-0.0001
L. verticillata	2010	13	IMG2476	*Anthophora*	24.46	0.0141	0.0532	0.0234	0.0385	0.0044	-0.0052	-0.0226	-0.0100	0.0128	0.0177	0.0183	-0.0025	0.0022	0.0107	0.0010	0.0001	-0.0047	-0.0025	0.0000	-0.0007	-0.0001	-0.0005	-0.0005	0.0005	-0.0016	0.0004	0.0007	0.0002	0.0004	-0.0002
L. verticillata	2010	14	IMG2480	*Anthophora*	29.50	0.1110	0.1070	-0.0296	-0.0265	-0.0038	0.0092	0.0028	-0.0178	-0.0080	-0.0022	0.0082	0.0091	-0.0022	-0.0052	-0.0019	-0.0004	0.0002	0.0007	-0.0018	-0.0012	0.0006	0.0004	-0.0004	-0.0008	0.0006	0.0006	0.0000	-0.0001	0.0001	0.0000
L. verticillata	2010	15	IMG2483	*Anthophora*	25.06	-0.0035	0.0571	-0.0176	-0.0197	0.0073	0.0102	0.0045	-0.0174	0.0024	0.0101	0.0097	-0.0027	0.0140	-0.0014	0.0018	-0.0031	0.0049	0.0024	-0.0005	0.0016	-0.0006	-0.0020	-0.0011	0.0003	0.0002	-0.0003	0.0000	-0.0005	0.0001	-0.0003
L. verticillata	2010	16	IMG2486	*Anthophora*	26.72	0.0291	0.0597	-0.0090	0.0337	0.0065	-0.0076	0.0006	-0.0049	0.0057	0.0178	0.0047	0.0002	0.0109	0.0079	-0.0053	-0.0039	-0.0031	0.0019	0.0013	-0.0005	-0.0015	-0.0002	-0.0004	0.0004	-0.0005	0.0010	0.0008	0.0001	0.0008	-0.0001
L. verticillata	2010	17	IMG2489	*Anthophora*	26.00	-0.0254	0.0769	-0.0028	0.0131	-0.0156	-0.0100	-0.0050	-0.0091	0.0019	0.0219	0.0095	-0.0071	0.0020	0.0059	0.0007	-0.0025	0.0021	-0.0002	0.0032	0.0000	0.0005	-0.0018	-0.0006	-0.0002	-0.0012	0.0002	0.0003	0.0001	0.0008	0.0000
L. verticillata	2012	18	IMG1554	*Anthophora*	25.01	0.0195	0.0127	-0.0036	0.0348	0.0318	-0.0022	0.0016	0.0168	-0.0395	0.0208	0.0081	-0.0025	0.0019	0.0018	-0.0044	-0.0024	-0.0002	-0.0054	0.0051	0.0000	-0.0005	0.0002	0.0011	0.0002	0.0014	-0.0002	-0.0011	0.0002	-0.0005	-0.0007
L. verticillata	2012	19	IMG1557	*Anthophora*	23.83	-0.0164	0.0563	0.0038	0.0012	0.0598	0.0158	0.0274	0.0170	-0.0186	0.0179	0.0180	0.0042	-0.0064	-0.0028	0.0084	0.0008	0.0003	0.0014	-0.0006	0.0010	-0.0021	-0.0003	0.0007	0.0007	0.0005	-0.0008	0.0002	0.0004	-0.0001	0.0000
L. verticillata	2012	20	IMG1560	*Anthophora*	23.26	0.0175	0.0230	0.0387	0.0210	0.0447	0.0166	-0.0067	0.0206	-0.0334	0.0093	0.0085	0.0055	-0.0029	0.0011	-0.0003	-0.0028	-0.0004	0.0003	-0.0031	0.0005	0.0010	0.0020	0.0005	0.0006	0.0002	-0.0003	-0.0004	-0.0003	-0.0005	-0.0001
L. verticillata	2012	21	IMG1563	*Anthophora*	25.82	-0.0212	0.0872	-0.0178	0.0153	0.0401	-0.0009	-0.0067	0.0095	-0.0108	0.0052	0.0161	0.0019	-0.0086	0.0003	0.0074	-0.0059	-0.0020	0.0015	-0.0035	0.0002	-0.0011	-0.0015	0.0007	0.0006	-0.0008	-0.0001	0.0000	0.0007	-0.0003	0.0000
L. verticillata	2012	22	IMG1566	*Anthophora*	25.11	0.0434	0.0368	0.0239	0.0397	0.0397	0.0025	0.0033	0.0046	-0.0232	0.0238	0.0069	0.0001	-0.0094	-0.0002	0.0049	-0.0021	-0.0049	-0.0001	0.0011	-0.0006	0.0005	0.0013	0.0001	-0.0001	-0.0003	-0.0004	-0.0004	0.0001	-0.0004	-0.0001
L. verticillata	2012	23	IMG1569	*Anthophora*	25.20	-0.0356	0.0596	0.0091	0.0317	0.0361	0.0044	-0.0047	0.0193	-0.0193	0.0137	0.0143	0.0010	-0.0051	0.0024	0.0061	-0.0023	-0.0023	0.0012	-0.0034	-0.0014	0.0009	-0.0007	-0.0003	0.0006	-0.0006	-0.0005	0.0000	0.0002	-0.0003	-0.0002
L. verticillata	2012	24	IMG1572	*Anthophora*	27.77	0.0139	0.0957	-0.0104	0.0035	0.0496	-0.0103	-0.0132	0.0024	-0.0046	0.0126	0.0110	0.0027	-0.0110	0.0032	0.0127	-0.0016	0.0007	0.0005	-0.0024	-0.0005	-0.0022	-0.0029	0.0014	-0.0007	-0.0007	-0.0003	-0.0001	0.0008	-0.0003	0.0002
L. verticillata	2012	25	IMG1575	*Anthophora*	27.43	-0.0250	0.1371	0.0032	0.0133	0.0258	-0.0363	-0.0191	0.0085	-0.0098	0.0011	0.0097	0.0070	0.0003	0.0090	0.0038	0.0017	0.0000	-0.0029	0.0020	0.0018	0.0014	0.0004	0.0000	-0.0015	-0.0008	-0.0005	-0.0001	-0.0001	0.0006	-0.0001
L. verticillata	2012	26	IMG1578	*Anthophora*	25.49	-0.0457	0.1004	-0.0071	0.0362	0.0009	-0.0014	0.0025	-0.0181	-0.0252	-0.0085	0.0077	0.0171	0.0015	-0.0052	0.0098	-0.0108	0.0016	-0.0032	0.0011	0.0077	-0.0012	0.0019	-0.0017	-0.0012	-0.0006	-0.0016	-0.0006	-0.0002	0.0008	-0.0001
L. verticillata	2012	27	IMG1581	*Anthophora*	26.32	0.0243	0.1445	-0.0074	0.0642	0.0209	-0.0020	-0.0215	-0.0051	-0.0001	0.0156	-0.0037	0.0044	-0.0003	0.0015	0.0020	0.0030	0.0032	0.0030	-0.0033	0.0006	0.0022	0.0002	-0.0003	0.0005	-0.0004	0.0000	-0.0002	-0.0005	-0.0003	0.0002

Supplementary Table 2. Morphometric data of pollinator specimens

Pollinator species	Individual No.	Collection No.	Photo No.	Pollinator group	Pollinator size (proboscids-*scutum*; mm)
Chalicodoma parietina	1	156JB10	P4171197	Megachilidae	15.49
Chalicodoma parietina	2	155JB10	P4171203	Megachilidae	14.52
Chalicodoma parietina	3	157JB10	P4171205	Megachilidae	14.18
Chalicodoma parietina	4	159JB10	P4171212	Megachilidae	12.87
Chalicodoma parietina	5	155PV10e	P4171216	Megachilidae	16.60
Chalicodoma parietina	6	155PV10b	P4171222	Megachilidae	14.22
Chalicodoma parietina	7	155PV10d	P4171228	Megachilidae	15.56
Chalicodoma parietina	8	79PV12(1)	P4171231	Megachilidae	14.96
Chalicodoma parietina	9	79PV12(2)	P4171235	Megachilidae	15.27
Chalicodoma parietina	10	155PV10a	P4171238	Megachilidae	14.76
Chalicodoma parietina	11	165PV10b	P5221368	Megachilidae	16.70
Chalicodoma parietina	12	165PV10c	P5221372	Megachilidae	18.05
Rhodanthidium sticticum	1	13JB10	P4101001	Megachilidae	12.16
Rhodanthidium sticticum	2	40JB10	P4101003	Megachilidae	11.27
Rhodanthidium sticticum	3	41JB10	P4101016	Megachilidae	12.93
Rhodanthidium sticticum	4	43JB10	P4101021	Megachilidae	10.29
Rhodanthidium sticticum	5	46JB10	P4101024	Megachilidae	12.67
Rhodanthidium sticticum	6	47JB10	P4101029	Megachilidae	11.03
Rhodanthidium sticticum	7	55JB10	P4101035	Megachilidae	11.10
Rhodanthidium sticticum	8	51JB10	P4101037	Megachilidae	11.40
Rhodanthidium sticticum	9	54JB10	P4101038	Megachilidae	12.52
Rhodanthidium sticticum	10	91JB10	P4101045	Megachilidae	12.93
Rhodanthidium sticticum	11	7JB11	P4101048	Megachilidae	12.61
Rhodanthidium sticticum	12	6JB11	P4101052	Megachilidae	12.39
Rhodanthidium sticticum	13	5JB11	P4101056	Megachilidae	12.44
Rhodanthidium sticticum	14	8JB11	P4101063	Megachilidae	10.95
Rhodanthidium sticticum	15	27JB12	P4101066	Megachilidae	11.56
Rhodanthidium sticticum	16	53PV10(b)	P4291270	Megachilidae	13.20
Rhodanthidium sticticum	17	53PV10(a)	P4291274	Megachilidae	12.49
Rhodanthidium sticticum	18	53PV10(2)	P4291275	Megachilidae	11.99
Rhodanthidium sticticum	19	53PV10(1)	P4291278	Megachilidae	12.92
Rhodanthidium sticticum	20	53PV10(8)	P4291280	Megachilidae	13.82
Rhodanthidium sticticum	21	53PV10(7)	P4291284	Megachilidae	12.80
Anthophora plumipes	1	75JB10	P4151152	*Anthophora*	17.89
Anthophora plumipes	2	76JB10	P4151155	*Anthophora*	16.62
Anthophora plumipes	3	77JB10	P4151162	*Anthophora*	16.24
Anthophora plumipes	4	81JB10	P4151169	*Anthophora*	17.48
Anthophora plumipes	5	85JB10	P4151174	*Anthophora*	17.11
Anthophora plumipes	6	15JB10	P4151185	*Anthophora*	17.07
Anthophora plumipes	7	28JB12	P4171193	*Anthophora*	17.93
Anthophora plumipes	8	87JB10	P4251253 y P4251255	*Anthophora*	16.39
Anthophora plumipes	9	86JB10	P451257 y P451258	*Anthophora*	13.68
Anthophora plumipes	10	26JB12	P4251259 y P4251261	*Anthophora*	15.43
Anthophora plumipes	11	48MF10	P5221374 Y P5221376	*Anthophora*	17.22
Anthophora plumipes	12	1 (Pozuelo de Alarcón, Madrid)	P5221378	*Anthophora*	16.18
Anthophora plumipes	13	2 (Orense, 31-3-72, M.L. Ortiz)	P5221379	*Anthophora*	19.18
Anthophora plumipes	14	3 (Collado Villalba, 17-v-70, M Cuesta)	P5221385	*Anthophora*	19.92
Anthophora plumipes	15	4 (Palma, 3-abril-1960)	P5221388 y P5221392	*Anthophora*	20.28
Anthophora plumipes	16	5 (Madrid, C.U., 16-IV-79, R. Outerelo)	P5221394	*Anthophora*	17.18
Anthophora plumipes	17	6 (J.M. Freire, Segovia, 8-8-74)	P5221397	*Anthophora*	17.88
Anthophora plumipes	18	7 (Doñana, 20-3-71, MA Comendador)	P5221400	*Anthophora*	18.16
Anthophora plumipes	19	8 (Palma, 3-abril-1960)	P5221404	*Anthophora*	21.45
Anthophora plumipes	20	9 (Piera Buena, 7-72, Esteban)	P5221409	*Anthophora*	17.85

Rhodanthidium sticticum	1	13JB10	P4101001	Megachilidae	12.16
Rhodanthidium sticticum	2	40JB10	P4101003	Megachilidae	11.27
Rhodanthidium sticticum	3	41JB10	P4101016	Megachilidae	12.93
Rhodanthidium sticticum	4	43JB10	P4101021	Megachilidae	10.29
Rhodanthidium sticticum	5	46JB10	P4101024	Megachilidae	12.67
Rhodanthidium sticticum	6	47JB10	P4101029	Megachilidae	11.03
Rhodanthidium sticticum	7	55JB10	P4101035	Megachilidae	11.10
Rhodanthidium sticticum	8	51JB10	P4101037	Megachilidae	11.40
Rhodanthidium sticticum	9	54JB10	P4101038	Megachilidae	12.52
Rhodanthidium sticticum	10	91JB10	P4101045	Megachilidae	12.93
Rhodanthidium sticticum	11	7JB11	P4101048	Megachilidae	12.61
Rhodanthidium sticticum	12	6JB11	P4101052	Megachilidae	12.39
Rhodanthidium sticticum	13	5JB11	P4101056	Megachilidae	12.44
Rhodanthidium sticticum	14	8JB11	P4101063	Megachilidae	10.95
Rhodanthidium sticticum	15	27JB12	P4101066	Megachilidae	11.56
Rhodanthidium sticticum	16	53PV10(b)	P4291270	Megachilidae	13.20
Rhodanthidium sticticum	17	53PV10(a)	P4291274	Megachilidae	12.49
Rhodanthidium sticticum	18	53PV10(2)	P4291275	Megachilidae	11.99
Rhodanthidium sticticum	19	53PV10(1)	P4291278	Megachilidae	12.92
Rhodanthidium sticticum	20	53PV10(8)	P4291280	Megachilidae	13.82
Rhodanthidium sticticum	21	53PV10(7)	P4291284	Megachilidae	12.80
Anthophora plumipes	1	75JB10	P4151152	*Anthophora*	17.89
Anthophora plumipes	2	76JB10	P4151155	*Anthophora*	16.62
Anthophora plumipes	3	77JB10	P4151162	*Anthophora*	16.24
Anthophora plumipes	4	81JB10	P4151169	*Anthophora*	17.48

Anthophora plumipes	5	85JB10	P4151174	*Anthophora*	17.11
Anthophora plumipes	6	15JB10	P4151185	*Anthophora*	17.07
Anthophora plumipes	7	28JB12	P4171193	*Anthophora*	17.93
Anthophora plumipes	8	87JB10	P4251253 y P4251255	*Anthophora*	16.39
Anthophora plumipes	9	86JB10	P451257 y P451258	*Anthophora*	13.68
Anthophora plumipes	10	26JB12	P4251259 y P4251261	*Anthophora*	15.43
Anthophora plumipes	11	48MF10	P5221374 Y P5221376	*Anthophora*	17.22
Anthophora plumipes	12	C.U.1 (Pozuelo de Alarcón, Madrid)	P5221378	*Anthophora*	16.18
Anthophora plumipes	13	C.U.2 (Orense, 31-3-72, M.L. Ortiz)	P5221379	*Anthophora*	19.18
Anthophora plumipes	14	C.U.3 (Collado Villalba, 17-v-70, M Cuesta)	P5221385	*Anthophora*	19.92
Anthophora plumipes	15	C.U.4 (Palma, 3-abril-1960)	P5221388 y P5221392	*Anthophora*	20.28
Anthophora plumipes	16	C.U.5 (Madrid, C.U., 16-IV-79, R. Outerelo)	P5221394	*Anthophora*	17.18
Anthophora plumipes	17	C.U.6 (J.M. Freire, Segovia, 8-8-74)	P5221397	*Anthophora*	17.88
Anthophora plumipes	18	C.U.7 (Doñana, 20-3-71, MA Comendador)	P5221400	*Anthophora*	18.16
Anthophora plumipes	19	C.U.8 (Palma, 3-abril-1960)	P5221404	*Anthophora*	21.45
Anthophora plumipes	20	C.U.9 (Piera Buena, 7-72, Esteban)	P5221409	*Anthophora*	17.85
Anthophora plagiata	1	16JB10	P4100948	*Anthophora*	15.83
Anthophora plagiata	2	61JB10	P4100952	*Anthophora*	18.11
Anthophora plagiata	3	62JB10	P4100955	*Anthophora*	19.30
Anthophora plagiata	4	64JB10	P4100963	*Anthophora*	18.16
Anthophora plagiata	5	45JB10	P4100969	*Anthophora*	15.13
Anthophora plagiata	6	67JB10	P4100973	*Anthophora*	16.66
Anthophora plagiata	7	26JB10	P4100979	*Anthophora*	18.41
Anthophora plagiata	8	22JB12	P4100998	*Anthophora*	19.11
Anthophora plagiata	9	66JB10	P4251263	*Anthophora*	17.26
Anthophora plagiata	10	19JB12	P4251265	*Anthophora*	19.20
Anthophora plagiata	11	21JB12	P42551266 y P4251269	*Anthophora*	17.55
Xylocopa violacea	1	4JB13	P5091296	*Xylocopa*	25.48
Xylocopa violacea	2	6JB13	P5091298	*Xylocopa*	24.59
Xylocopa violacea	3	C.U.1 (Madrid, 25-5-73, J.Baeza)	P5091300	*Xylocopa*	24.55
Xylocopa violacea	4	C.U.2 (Aranjuez, IX-1972, A. Fernández	P5091303	*Xylocopa*	22.62
Xylocopa violacea	5	C.U.3 (C.U., Madrid, 11-5-71, E. de Juana)	P5091305	*Xylocopa*	23.50
Xylocopa violacea	6	C.U.4 (Madrid, 19-V-73, Díez)	P5091306	*Xylocopa*	23.23
Xylocopa violacea	7	C.U.5 (Fontanar, Guadalajara, 7-IV-77, M. G	P5091309 y P5091310	*Xylocopa*	24.49
Xylocopa violacea	8	C.U.6 (C.U., 29-4-72)	P5091313 y P5091314	*Xylocopa*	22.14
Xylocopa violacea	9	C.U.7 (Madrid, 30-4-72, Fdez. C.)	P5091318 y P5091319	*Xylocopa*	23.76
Xylocopa violacea	10	C.U.8 (Madrid, 29-4-72, García)	P5091321 y P5091324	*Xylocopa*	21.78
Xylocopa violacea	11	C.U.9 (Villaviciosa, 18-4-72, Cabrera)	P5091326	*Xylocopa*	20.21
Xylocopa violacea	12	C.U.10 (C. Caminos, Madrid, 10-4-72, P.M.Ca	P5091332	*Xylocopa*	22.04
Xylocopa violacea	13	C.U.11(Canilejas, Madrid, VII-1973, M.L.Sa	P5091337	*Xylocopa*	23.27
Xylocopa violacea	14	C.U.12 (C.U., Madrid, 3-V-71, Mamolar)	P5091339	*Xylocopa*	21.64
Xylocopa violacea	15	C.U.13 (Moratalaz, 8-V-77, Carmen López)	P5091343	*Xylocopa*	21.82
Xylocopa violacea	16	C.U.14 (Madrid, 8-5-77, Manuel Sánchez)	P5091350	*Xylocopa*	21.76
Xylocopa violacea	17	C.U.15 (C.U., Madrid, 6-4-72, E. Cubas)	P5091351	*Xylocopa*	21.76
Xylocopa violacea	18	C.U.16 (C.Universit., 6-V-70, F. González)	P5091357	*Xylocopa*	22.96
Xylocopa violacea	19	C.U.17 (Canillejas, 25-X-70, J.R.Elizalde)	P5091360	*Xylocopa*	20.38
Xylocopa violacea	20	C.U.18 (Guadalajara, 5-72, R. Yagüe)	P5091363	*Xylocopa*	21.86
Xylocopa violacea	21	C.U.19 (Casa de Campo, 16-IV-74, A.A. Luque	P5091365	*Xylocopa*	19.46
Chalicodoma pyrenaica	1	60JB10	P4151070	Megachilidae	11.38
Chalicodoma pyrenaica	2	50JB10	P4151072	Megachilidae	12.74
Chalicodoma pyrenaica	3	52JB10	P4151073	Megachilidae	12.92
Chalicodoma pyrenaica	4	53JB10	P4151078	Megachilidae	13.77
Chalicodoma pyrenaica	5	27JB10	P4151081	Megachilidae	13.75
Chalicodoma pyrenaica	6	20JB10	P4151086	Megachilidae	12.07
Chalicodoma pyrenaica	7	28JB10	P4151089	Megachilidae	12.88
Chalicodoma pyrenaica	8	19JB10	P4151093	Megachilidae	13.08
Chalicodoma pyrenaica	9	22JB10	P4151099	Megachilidae	13.70
Chalicodoma pyrenaica	10	23JB10	P4151102	Megachilidae	13.74
Chalicodoma pyrenaica	11	24JB10	P4151108	Megachilidae	13.96
Chalicodoma pyrenaica	12	11JB10	P4151112	Megachilidae	12.69
Chalicodoma pyrenaica	13	105JB10	P4151116	Megachilidae	11.48
Chalicodoma pyrenaica	14	136JB10	P4151123	Megachilidae	12.57
Chalicodoma pyrenaica	15	98JB10	P4151126	Megachilidae	12.30
Chalicodoma pyrenaica	16	138JB10	P4151129	Megachilidae	12.77
Chalicodoma pyrenaica	17	99JB10	P4151134	Megachilidae	12.10
Chalicodoma pyrenaica	18	101JB10	P4151138	Megachilidae	11.10
Chalicodoma pyrenaica	19	117JB10	P4151141	Megachilidae	12.73
Chalicodoma pyrenaica	20	3JB12	P4151145	Megachilidae	13.27
Anthophora crassipes	1	125JB10	P4241241	*Anthophora*	11.10
Anthophora crassipes	2	150JB10	P4241244	*Anthophora*	11.26
Anthophora crassipes	3	108JB10	P4241247	*Anthophora*	13.99
Anthophora crassipes	4	142JB10	P4241251	*Anthophora*	13.55
Anthophora crassipes	5	74PV09	P4291286 y P4291289	*Anthophora*	13.48
Anthophora crassipes	6	82PV09	P4291291 y P42919	*Anthophora*	14.60

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Past and future demographic dynamics of alpine species: limited genetic consequences despite dramatic range contraction in a plant from the Spanish Sierra Nevada

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Abstract

Anthropogenic global climate change is expected to cause severe range contractions among alpine plants. Alpine areas in the Mediterranean region are of special concern because of the high abundance of endemic species with narrow ranges. This study combined species distribution models, population structure analyses and Bayesian skyline plots to trace the past and future distribution and diversity of *Linaria glacialis*, an endangered narrow endemic species that inhabits summits of Sierra Nevada (Spain). The results showed that: (i) the habitat of this alpine-Mediterranean species in Sierra Nevada suffered little changes during glacial and interglacial stages of late Quaternary; (ii) climatic oscillations in the last millennium (Medieval Warm Period and Little Ice Age) moderately affected the demographic trends of *L. glacialis*; (iii) future warming conditions will cause severe range contractions; and (iv) genetic diversity will not diminish at the same pace as the distribution range. As a consequence of the low population structure of this species, genetic impoverishment in the alpine zones of Sierra Nevada should be limited during range contraction. We conclude that maintenance of large effective population sizes via high mutation rates and high levels of gene flow may promote the resilience of alpine plant species when confronted with global warming.

Keywords: alpine-Mediterranean, Bayesian skyline plots, conservation, climate change, effective population size, *Linaria*, species distribution modelling

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Introduction

Increasing evidence suggests that anthropogenic global climate change (GCC) is changing the distribution and abundance of organisms (Rosenzweig *et al.* 2008). With further expected GCC, accurate scientific predictions of its effects on biodiversity will be required for conservation planning. Mountains contain range-restricted ecosystems where human impacts have been limited compared with lowland regions. The stratified vegetation belts of mountains contain small hotspots of biodiversity at a fragile equilibrium. In particular, the alpine vegetation belt includes discrete domains where small perturbations in global processes can produce large quantifiable changes (Diaz *et al.* 2003). Therefore, the alpine life zone provides a unique opportunity for monitoring climate impacts (Pauli *et al.* 2004). Studies involving the effect of global warming on alpine species have detected a general pattern of range contraction, as species move upward in elevation (Pauli *et al.* 1996; Thuiller *et al.* 2005; Walther *et al.* 2005; Engler *et al.* 2011; Gottfried *et al.* 2012). Thus, in a global warming scenario, the habitats of alpine vegetation could be restricted drastically, which might result in rapid extinctions (Parmesan 2006). The extinction risk of species is even higher on Mediterranean summits, where the highest levels of biodiversity and endemism are found when compared with other European mountains (Pauli *et al.* 2012).

The Mediterranean Basin is one of the world's major centres of plant diversity (Médail & Quézel 1997). The

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palaeogeologic Baetic-Rifan complex, located in the western Mediterranean, includes mountains of both southern Spain and northwestern Africa and is considered one of the most important biodiversity hotspots within the Mediterranean Basin (Médail & Quézel 1997, 1999). In the Baetic range (Spain), the Sierra Nevada mountains (see Fig. 1) harbour an exceptionally unique flora with >50% endemics *sensu lato*, that is, strict endemics plus Baetic-North African endemics plus Iberian endemics (Gomez-Campo *et al.* 1984). Around 2100 plant species are present in Sierra Nevada. In the alpine vegetation belt (also called cryoromediterranean belt) there are 185 species, of which 55 (~30%) are endemics *sensu stricto* (Fernández Calzado & Molero Mesa 2011). As a result of comparing the landscape descriptions of naturalists of the 18th and 19th centuries, it was inferred that the lower limit of the alpine vegetation belt has moved upwards in elevation (reviewed in Gómez-Ortiz *et al.* 2009). This indicates that this vegetation belt has experienced a major range reduction during the last ca. 150 years, which may have started at the end of the Little Ice Age (LIA) period.

The Sierra Nevada summits are considered one of the best areas to evaluate climate change in Europe (Benito *et al.* 2011; Global Observation Research Initiative in Alpine Environments (GLORIA), http://www.gloria.ac.at/; Sierra Nevada Observatory for Global Change (SNOGC), http://linaria.obsnev.es/). For instance, climatic projections for the 21st century suggest a relatively uniform increase in temperatures in the region. For the whole mountain range, the mean annual temperature is predicted to be increased by 4.4–7 °C under the least favourable scenario (a2 of IPPC) and by 2.3–5.6 °C under the most favourable one (b2 of IPCC), during the period 2010–2100 (Benito *et al.* 2011). This suggests that even in the most optimistic scenario, there is a serious risk of increased climatic stress in Sierra Nevada, which might be even greater in summit areas (see Fig. S3, Supporting information).

In the last two decades, species distribution models (SDMs) have been increasingly used for conservation purposes (see Benito *et al.* 2009 for an example in *Linara*), and particularly to predict the impacts of GCC on biodiversity (Thomas *et al.* 2004; Guisan & Thuiller 2005). SDMs are useful tools for predicting future extinction risk caused by habitat contraction. The genomes of species contain historical signatures and the raw material to adapt future changes, so knowledge on population genetic structure, gene flow and demographics, together with SDMs is also required for more reliable evaluations (Scoble & Lowe 2010; Hoffmann & Sgro 2011; Jay *et al.* 2012; Pfenninger *et al.* 2012; Pauls *et al.* 2013). In particular, dispersal-limited species with highly structured genetic diversity may be more vulnerable to diversity loss than nearly panmictic species because the genetic diversity of the latter will be spatially distributed throughout the species range (Leblois *et al.* 2006).

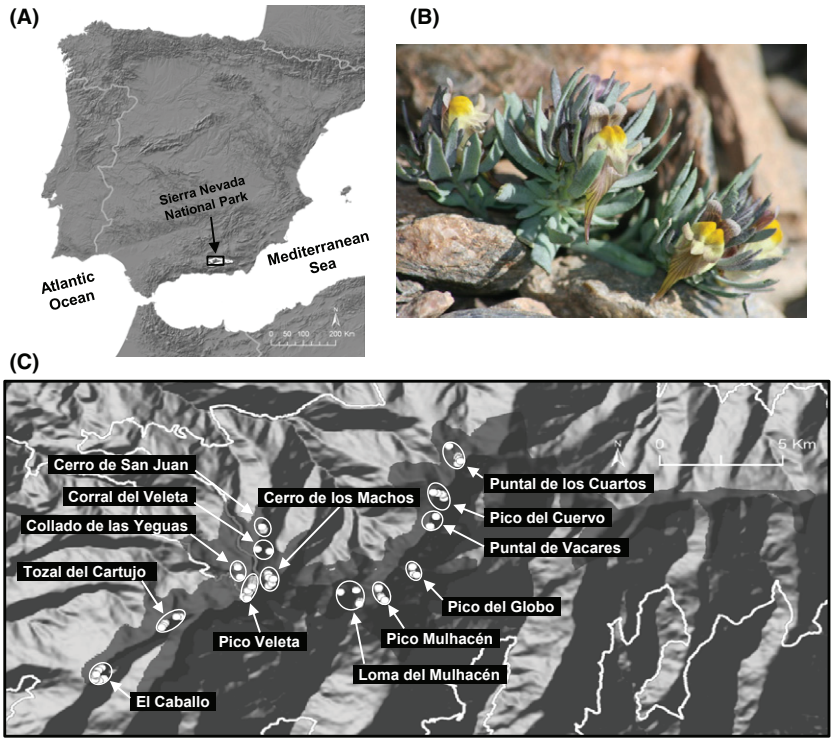

Fig. 1 (A) Location of the study area. (B) Photograph of *Linaria glacialis* Boiss. (C) Sampling sites (white ovals), individuals sampled (white dots) and potential distribution range of *L. glacialis* (in shaded grey, see also Fig. 6E). The white line indicates the limits of the Sierra Nevada National Park.

There have been significant advances in the application of molecular markers for understanding population genetic diversity dynamics and historical demography in the last years, such as Bayesian skyline plot methods (see a review in Ho & Shapiro 2011). Nevertheless, population genetic analyses and SDMs have rarely been used simultaneously to assess past population dynamics (but see Dépraz *et al.* 2008; Cordellier & Pfenninger 2009; Bisconti *et al.* 2011; Espíndola *et al.* 2012). In addition, these two approaches have seldom been combined to make past inferences and future predictions (but see Espíndola *et al.* 2012).

The present study used *Linaria glacialis* Boiss. as a model species to infer past and future distribution range shifts and demographic dynamics within the alpine vegetation belt of Sierra Nevada. This species is a well-suited model plant because of its: (i) endemic condition, (ii) well-documented evolutionary context (Blanco-Pastor *et al.* 2012; Fernández-Mazuecos *et al.* 2013) and (iii) distribution at the highest summits of Sierra Nevada (>3000 m). The specific objectives of the present study were: (i) to identify the current population genetic structure of *L. glacialis*, (ii) to reconstruct the changes in species range during the Quaternary, (iii) to track past demographic trends using a Bayesian coalescent approach, (iv) to unravel future environmental suitability by projecting SDMs to four different climate warming scenarios and (v) to project future changes in genetic diversity (effective population sizes, N_e) linked to future SDM projections. The ultimate aim was to assess the severity of changes in the levels of genetic diversity in a range contraction scenario driven by climate change.

Materials and methods

Sampling and DNA sequencing strategy

We collected 103 *L. glacialis* individuals during two field trips (July 2009/2010) to the summits of Sierra Nevada (see Appendix S1 for further details of the study species and the study area). We collected material in 13 localities distributed throughout the potential distribution of the species (Fig. 1). After collection, individuals were dried in silica gel. DNA extraction, amplification and sequencing were carried out as in Blanco-Pastor *et al.* (2012). We sequenced two unlinked loci in most *L. glacialis* individuals and one *L. verticillata* individual used as outgroup (*L. glacialis* data set; see Tables S1 and S2, Supporting information): (i) the low-copy nuclear gene AGT1 (partial exon and partial intron sequences) and (ii) two linked plastid regions (*rpl*32-*trn*L^{UAG}, *rps*162F2-*trn*K^{UUU}) (ptDNA). The outgroup species was selected following a species tree analysis carried out with two individuals of *L. glacialis* and 15 additional individuals of closely related species (species-level data set; see Tables S1 and S2, Appendix S1, and Fig. S1, Supporting information).

Deciphering of haplotypes from unphased genotypes and test for recombination

Numerous ambiguities were found in the unphased data of the low-copy nuclear gene (AGT1) of the *L. glacialis* data set. To obtain haplotypes from unphased genotypes, we designed 12 allele-specific sequencing primers (ASP) following Scheen *et al.* (2011) (see Table S3, Supporting information). The ASPs (two ASPs employed per unphased sequence) resolved all ambiguities except for the first ambiguity of 48 sequences which could not be resolved due to its proximity to the primer bind. This allowed us to rule out the presence of paralogous copies in the AGT1 sequences of the *L. glacialis* data set. To solve the remaining ambiguities located in the primer binding sites of ASPs, we used the Bayesian method of PHASE 2.1 (Stephens *et al.* 2001; Stephens & Donnelly 2003), as implemented in DnaSP v5 (Librado & Rozas 2009) with default parameters. When phased sequences were not statistically supported (PP < 0.9), they were excluded from subsequent analyses. This haplotype gathering procedure allowed us to obtain accurate allelic data that were used in subsequent analyses.

Recombination was tested within the AGT1 alignment of the *L. glacialis* data set using the software RDP 3.44 (Martin *et al.* 2010). We used the same methods and parameter settings as in Blanco-Pastor *et al.* (2012).

Sequence diversity and tests for neutrality

Genetic diversity for the AGT1 and ptDNA loci of *L. glacialis* was estimated using the haplotype number, the haplotype diversity [H_d, defined as the probability that two randomly chosen haplotypes in the sample are different (Nei 1987)], the number of segregating sites and the nucleotide diversity [π, defined as the per site average number of nucleotide differences between pairs of sequences (Nei 1987)].

In order to examine evidence for selection in the amplified loci, neutrality tests were performed on sequence data as implemented in the program DnaSP v.5 (Librado & Rozas 2009). First, we calculated Tajima's *D* (Tajima 1989), which is based on the differences between the number of segregating sites and the average number of nucleotide differences between pairs of sequences (k). Tajima's *D* is expected to be close to zero under neutrality. Positive values represent an excess of intermediate frequency alleles and may be due to balancing selection or population contraction,

while negative values indicate an excess of low-frequency alleles and can represent positive selection or population expansion.

The Fu and Li's *D* and *F* tests (Fu & Li 1993) were additionally performed to evaluate neutrality. Fu and Li's *D* statistic is based on the difference between η_e, the total number of mutations in external branches of a gene genealogy (constructed here using *L. verticillata* as outgroup, see Fig. S1, Supporting information) and η, the total number of mutations. The *F* statistic is the normalized difference between η_e and *k*. The mutations in external branches of a genealogy represent recent mutations. An excess of recent mutations may indicate negative selection (low frequency of novel deleterious alleles) or positive selection (high frequency of an old advantageous allele). A deficiency of recent mutations may indicate long-term balancing selection. Significance of Tajima's and Fu and Li's statistics was evaluated using neutral coalescent simulations with 10 000 replicates. Simulations were based on the observed number of segregating sites, assuming no recombination and with a significance level fixed to 0.05.

Population genetic structure

To identify the population genetic structure of *L. glacialis*, we conducted a spatial analysis of molecular variance (samova) (Dupanloup *et al.* 2002) implemented in the software SAMOVA 1.0. This method defines groups of populations (panmictic units) that are maximally differentiated from each other in a geographically homogeneous environment. Without a priori assumptions of population groupings, it maximizes the proportion of genetic variance among *K* groups. Genetic variance is assessed with the F_{CT} statistic, which is in turn defined by pairwise differences of the nucleotide sequences. Analyses with all possible *K* groups (2–12) were carried out with 100 simulated annealing runs. Analyses were performed independently for the AGT1 and the ptDNA loci to obtain two independent *K* groupings with maximized F_{CT} values.

Differentiation among the populations defined by the SAMOVA analysis of the most variable locus (AGT1; 10 populations, see below) was assessed by two methods. First, the fixation index F_{ST} (Wright 1943; Nei 1973) was calculated with ARLEQUIN v.3.5 (Excoffier & Lischer 2010). Then, we used GENODIVE v. 2.0b23 (Meirmans & Van Tienderen 2004) to calculate Jost's *D* statistic (Jost 2008), an index of population differentiation that is independent of the amount of within-population diversity and is less affected by mutation rates.

We also obtained the genealogical relationships among ptDNA haplotypes using statistical parsimony as implemented in TCS v1.21 (Clement *et al.* 2000). The maximum number of differences resulting from single substitutions among haplotypes was calculated with 95% confidence limits. Gaps were coded and treated as fifth character, but we excluded gaps generated by microsatellite length differences because of their high level of homoplasy. As much higher variation was found in the AGT1 alignment of the *L. glacialis* data set, the statistical parsimony network for this gene displayed disconnected lineages. For that reason, we alternatively conducted a gene tree analysis in MRBAYES 3.1.2 (Huelsenbeck & Ronquist 2001). The exon and intron regions of the AGT1 alignment were set as two independent partitions with different substitution models as inferred in JMODELTEST 0.1.1 (Posada 2008). We included one individual of *L. verticillata* as outgroup when constructing the TCS ptDNA haplotype network and the MRBAYES AGT1 phylogenetic tree.

SAMOVA analysis is based on allele frequencies and assumes that the processes giving rise to population structure have reached a dynamic equilibrium. In order to evaluate the optimal SAMOVA population structure under a more realistic scenario (nonequilibrium model with migration, mutation and fluctuating population sizes), we analysed the data with MIGRATE-n v.3.3.1 (Beerli & Palczewski 2010). MIGRATE-n allows the comparison of nested models of gene flow in a Bayesian framework by calculating Bayes factors. It includes an alternative method of estimating marginal likelihoods (mL) (thermodynamic integration) that has been shown to be more effective – it is less affected by prior distributions and has less variance – than the commonly used harmonic mean estimator (Lartillot & Philippe 2006). We compared the three maximally differentiated population groupings obtained in SAMOVA using AGT1 data (*K* = 2, 9 and 10; see below) against a model that considers *L. glacialis* to be formed by a single panmictic population. Natural log Bayes factors (LBF) were calculated via LBF = 2(lnmL(model_1)-lnmL(model_2)). Following Kass & Raftery (1995), LBF values smaller than −2 would suggest preference for model 2 while values larger than 2 would suggest preference for model 1. Model probabilities were calculated as follows:

$$\text{Prob}\,(\text{model}_i) = \frac{mL_{\text{model}_i}}{\sum_j^n mL_{\text{model}_j}} \quad \text{(Beerli 2012b)} \qquad (3)$$

See Supplementary data 1 for detailed parameter settings of MIGRATE-n analyses.

Population size history

To investigate the demographic history of *L. glacialis* we performed a coalescent-based Bayesian skyline plot analysis (BSP; Drummond et al. 2005). The BSP method

simultaneously estimates the evolutionary rate, substitution model parameters, phylogeny and ancestral population dynamics by using Markov chain Monte Carlo (MCMC) sampling. This analysis therefore estimates credibility intervals that represent the combined phylogenetic and coalescent uncertainty. Analysis was carried out in BEAST v.1.7.2 (Drummond *et al.* 2012) with the information of the two regions of AGT1, the most variable locus of the *L. glacialis* data set. Exon and intron regions were linked for the partition tree, but unlinked for the clock model and site model. In order to set an upper bound for the root age of *L. glacialis* samples, we included a treeModel.rootHeight prior with truncated-normal distribution (mean 0.096 Ma, standard deviation 0.096, lower limit = 0, upper limit = 0.25 Ma), derived from the divergence time between *L. glacialis* and its sister species (*L. verticillata*) as estimated in the species tree analysis of the species-level data set (see Fig. S1, Supporting information). The high variability of the AGT1 locus allowed us to choose ten groups of coalescent intervals for the plot smoothing, which we considered appropriate given the narrow credible intervals and the good convergence of chains obtained (Heled & Drummond 2008). Remaining priors for the analysis were set as in Blanco-Pastor *et al.* (2012) except for the clock models. In this analysis, a strict molecular clock was enforced, as it is generally a good approximation for analyses at the population level (Yang 2006) and because, by simplifying the coalescent model, it helps convergence of chains. Also, the AGT1 mean.rate parameter was modified as an exploratory analysis showed that the upper bound of the prior uniform distribution previously used in Blanco-Pastor *et al.* (2012) (5×10^{-2} s/s/Ma) was too low for the *L. glacialis* data set (posterior values reaching the upper bound). Therefore, we increased this upper limit up to 5 s/s/Ma. To obtain an appropriate tree rooting, we constrained the two sister groups of *L. glacialis* as obtained in the MRBAYES analysis (see Fig. 3A), which was rooted with *L. verticillata* accessions. Analysis was carried out with four MCMC runs for 100 million generations and a sample frequency of 10 000. Analysis with TRACER v.1.5 (Rambaut & Drummond 2009) confirmed appropriate mixing. Additionally, with the aim to detect further bottlenecks that were not observed in the BSP analysis, an extended Bayesian skyline plot (EBSP) (Heled & Drummond 2008) was also performed by including both AGT1 and ptDNA loci (see Appendix S1 for further details).

Past, present and future distribution under climatic change scenarios

We performed species distribution modelling (SDM) to evaluate the potential range of *L. glacialis* under present climatic conditions and to project it to past Quaternary climatic stages and future climatic projections under GCC. We employed the maximum entropy algorithm, as implemented in MAXENT v3.3 (Phillips *et al.* 2006) because it is appropriate for presence-only data, and its performance has been shown to be consistently competitive in comparison with other methods (Elith *et al.* 2006). This algorithm models the probability distribution of species presences based on the maximum entropy principle (Jaynes 1957), subject to a set of constraints based on environmental variables.

For projections to past conditions, we first retrieved a set of 19 bioclimatic variables under current conditions (1950–2000 period) from the WorldClim 1.4 website (www.worldclim.org; Hijmans *et al.* 2005). The WorldClim layers of bioclimatic variables were clipped to the extent of the Iberian Peninsula. After a correlation analysis in a random sample of 1000 points of the Iberian Peninsula, a reduced set of seven uncorrelated variables was selected: isothermality (bio3), temperature seasonality (bio4), maximum temperature of warmest month (bio5), minimum temperature of coldest month (bio6), precipitation of wettest month (bio13), precipitation of driest month (bio14) and precipitation seasonality (bio15) (Fernández-Mazuecos & Vargas 2013). The *L. glacialis* distribution model under current conditions was projected to the last interglacial (LIG; ca. 120 kya – 140 kya), last glacial maximum (LGM; ca. 21 kya) and mid-Holocene (ca. 6 kya). We used layers at ~1 km spatial resolution for present time, LIG and mid-Holocene periods and at ~5 km for LGM period, available from WorldClim 1.4. The palaeoclimatic layers of LIG were derived from Otto-Bliesner *et al.* (2006). Layers of LGM were obtained via the Paleoclimate Modelling Intercomparison Project phase 2 (Braconnot *et al.* 2007) and were based on simulations from two general atmospheric models: the Community Climate System Model version 3 (CCSM3; Collins *et al.* 2006) and the Model for Interdisciplinary Research on Climate (MIROC; Hasumi & Emori 2004). For the mid-Holocene period, we used layers simulated under the ECHAM3 model (Roeckner *et al.* 1992).

For projections to future conditions, we used current and future layers of Sierra Nevada available in the Linaria v.1.0 online database of the SNOGC (http://linaria.obsnev.es/). Future layers were obtained under four AGCC scenarios (ECHAM4-a2, ECHAM4-b2, CGCM2-a2 and CGCM2-b2). Three yearly 100-m resolution layers were available: (i) average maximum temperature, (ii) average minimum temperature and (iii) average precipitation. We selected the two temperature layers for 5-year intervals from 2015 to 2100 (precipitation was discarded as spatial models of rainfall in Sierra Nevada were considered problematic (see Benito *et al.* 2011)). Additionally, as *L. glacialis* is only present in

schistose rocky slopes, we used the Andalusian soil map (CMA 2005) to account for the edaphic complexity in current and future models, assuming that edaphology of the region will not suffer major changes in the next 90 years. To evaluate the effect of the inclusion of the edaphology variable in the model, we additionally performed a second analysis without this layer and compared results.

Species occurrence data were based on 103 GPS georeferenced individuals obtained during fieldtrips in July 2009/2010 (Table S2, Supporting information). Replicate runs (10) were performed by using the subsampling run type, and model evaluation was performed using the AUC test of MAXENT, with the 25% of presence records as the evaluation data set. Final habitat-suitability models were converted from continuous (the logistic MAXENT output) to binary (presence–absence output) using the maximum training sensitivity plus specificity logistic threshold (Jiménez-Valverde & Lobo 2007). For projections of habitat suitability, it was assumed that the ecological requirements of *L. glacialis* remain stable through time (Nogués-Bravo 2009).

Impact of climatic warming on the effective population size of L. glacialis

The population genetic parameter theta ($\theta_w = x\mu N_e$) (Watterson's θ; Watterson 1975) – where x is the heredity constant, μ is the mutation rate per nucleotide per generation and N_e is the effective population size – leads to an expectation of the amount of genetic diversity in a population. With a known mutation rate and an estimate of θ_w, we can then calculate the effective population size (N_e) of the population. We estimated projections of N_e between the years 2015 and 2100 at nine time slices corresponding to the subsequent persistence of the species in eleven, nine, seven, six, five, four, three, two and one localities. Species persistence was derived from MAXENT projections under the four climate warming scenarios. For each calculation of future N_e, the sequences of individuals collected at the projected nonsuitable areas were eliminated from the data matrices. For simplification, areas were defined as the putative (adjacent) population groupings supported by the SAMOVA analysis of the AGT1 locus (hereafter subpopulations, see below). Future N_e for each locus were obtained via $N_e = \theta_w/\mu$; θ_w values were derived from nine independent MIGRATE-n analyses corresponding with the nine time slices mentioned above with different inheritance scalars (x) for each locus as indicated in Beerli (2012a) (the ptDNA locus was used as reference) (see Data S1 and S2 for details). The mutation rate of each locus (μ) was derived from the EBSP analysis. In order to account for uncertainty in the N_e estimations, we considered the upper and lower bounds of the 95% highest posterior density (HPD) interval of θ_w and μ parameters as obtained in MIGRATE-n and EBSP analyses, respectively. N_e estimates were calculated considering a 1-year generation time. See Data S2 (Supporting information) for detailed parameter values of MIGRATE-n analyses.

Results

Haplotype data gathering and recombination test

The *L. glacialis* data set comprised 184 AGT1 and 101 ptDNA sequences (including outgroup). Recombination was not detected in the AGT1 alignment by any of the five methods used. Lack of recombination allowed us to analyse sequences without further modifications (i.e. discarding recombinant regions).

Genetic diversity and neutrality tests

Estimates of sequence diversity and neutrality tests can be found in Table 1. Number of haplotypes, haplotype diversity, number of segregating sites and nucleotide diversity were higher in the nuclear gene AGT1 when compared with the plastid locus. Tajima's, and Fu and Li's test did not identify any departures from neutrality with *P*-values above 0.05 when coalescent simulations were computed.

Population genetic structure

The SAMOVA analyses using the AGT1 locus allowed for the identification of 10 subpopulations as the grouping

Table 1 Diversity measures and neutrality tests carried out in AGT1 and ptDNA sequences

					Tajima's	Fu and Li's	
Locus	N_h	H_d	S	π	D	D	F
AGT1	43	0.932	72	3.54×10^{-2}	1.20 Ns	0.40 Ns	0.93 Ns
ptDNA	10	0.673	7	1.22×10^{-3}	5.40×10^{-2} Ns	−0.579 Ns	−0.432 Ns

N_h, number of haplotypes (gaps were excluded); H_d, haplotype diversity; S, segregating sites; π, nucleotide diversity; significance codes: Ns, not significant.

Table 2 Spatial analysis of molecular variance (samova) of AGT1 and ptDNA loci of *Linaria glacialis*. F_{CT} values and percentage of variation representing the among-group, among-population and the within-population levels of variation for the best-clustering option obtained for each value of *K*

			Percentage of variation		
K	F_{CT}	*P*-value	Among groups	Among populations within groups	Within populations
AGT1					
2	0.062	(*P* = 0.000)	6.19	−3.36	97.17
3	0.057	(*P* = 0.000)	5.74	−3.94	98.2
4	0.056	(*P* = 0.000)	5.65	−4.42	98.77
5	0.054	(*P* = 0.000)	5.41	−5.6	100.19
6	0.053	(*P* = 0.000)	5.29	−5.48	100.19
7	0.056	(*P* = 0.000)	5.65	−6.39	100.74
8	0.055	(*P* = 0.000)	5.51	−6.06	100.56
9	0.062	(*P* = 0.000)	6.2	−7.21	101.01
10	0.066	(*P* = 0.000)	6.59	−7.78	101.19
11	0.056	(*P* = 0.001)	5.59	−6.8	101.21
12	0.057	(*P* = 0.009)	5.58	−6.85	101.57
ptDNA					
2	0.394	(*P* = 0.000)	39.41	19.54	41.05
3	0.392	(*P* = 0.000)	39.19	18.55	42.26
4	0.389	(*P* = 0.000)	38.89	16.15	44.96
5	0.395	(*P* = 0.000)	39.52	5.76	54.72
6	0.386	(*P* = 0.000)	38.6	5.29	56.12
7	0.379	(*P* = 0.000)	37.9	5.48	56.62
8	0.376	(*P* = 0.000)	37.65	1.02	61.33
9	0.382	(*P* = 0.000)	38.19	0.18	61.63
10	0.383	(*P* = 0.000)	38.31	−2.23	63.92
11	0.396	(*P* = 0.000)	39.65	−4.18	64.53
12	0.429	(*P* = 0.008)	42.95	−7.69	64.75

with highest genetic variance (F_{CT} = 0.066, *P* = 0.00, Table 2 and Fig. 2A). This grouping was formed by arranging six sampling sites into three groups: two groups of two adjacent locations (Collado de las Yeguas-Corral del Veleta; Pico del Cuervo-Puntal de los Cuartos; Fig. 2A, see also Fig. 1C) and one group of two nonadjacent locations (Cerro de San Juan-Loma del Mulhacén; Fig. 2A, see also Fig. 1C). When using the ptDNA locus, SAMOVA identified 12 subpopulations from 13 sampling sites as the grouping with highest F_{CT} (F_{CT} = 0.429; *P* = 0.008, Table 2 and Fig. 2B). Only two (nonadjacent) sampling sites were grouped (El Caballo, Loma del Mulhacén; Fig. 2B). Overall, SAMOVA analyses indicated a lack of genetic structure as (i) *K* values with highest F_{CT} were very high (12 in ptDNA and 10 in AGT1 from 13 sampling sites) and (ii) the percentage explained by the within-population variation was always higher than that explained by the among-group variation, which was particularly evident in the AGT1 locus (Table 2). Population differentiation, as measured by pairwise F_{ST} values (Table 3), was extremely low according to the AGT1 locus and presented low to medium values according to the ptDNA locus. It has been argued that F_{ST} values can be dependent on the within-population diversity (similarity among populations can be wrongly interpreted when the within-population diversity is high), and that comparisons over loci with different mutation rates can yield extremely different values (Jost 2008; Meirmans & Hedrick 2011). In those cases, Jost's *D* (Jost 2008) is a better statistic to measure population differentiation as it is not affected by within-population diversity. The estimated values of this statistic were extremely low in both data sets

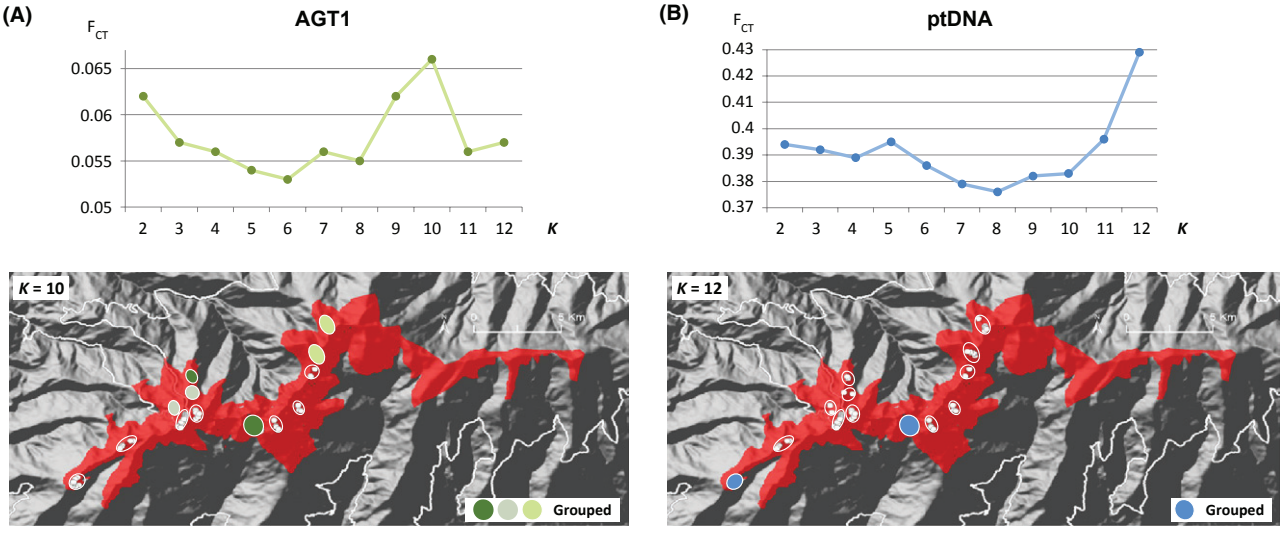

Fig. 2 Spatial analysis of molecular variance (samova) of AGT1 (A) and ptDNA (B) loci of *Linaria glacialis*. Graph of F_{CT} values representing the among-group variation for the best clustering obtained under each value of K. Maps represent the best clustering determined by the highest F_{CT} values. Potential distribution range is shown in shaded red (see also Fig. 6E).

Table 3 Measurements of population structure obtained for AGT1/ptDNA. The F_{ST} (below diagonal) and Jost's D (above diagonal) statistics of subpopulation pairs were obtained. Subpopulations were determined according to the best grouping of SAMOVA analysis of the AGT1 locus (10 subpopulations)

	1	2	3	4	5	6	7	8	9	10
1	—	0.037/0.077	0.021/0.042	0.042/0.086	0.039/0.062	0.024/0.051	0.026/0.056	0.028/0.061	0.033/0.089	0.027/0.045
2	−0.041/0.318*	—	0.032/0.062	0.056/0.103	0.049/0.081	0.036/0.067	0.036/0.072	0.037/0.075	0.047/0.103	0.039/0.061
3	−0.039/0.073	−0.010/0.602*	—	0.038/0.070	0.034/0.046	0.017/0.036	0.021/0.040	0.023/0.046	0.026/0.074	0.022/0.030
4	−0.005/0.200	0.032/0.120	0.028/0.459*	—	0.053/0.089	0.041/0.078	0.042/0.082	0.044/0.087	0.053/0.115	0.034/0.071
5	−0.014/−0.048	−0.014/0.269*	0.019/0.103*	−0.008/0.015	—	0.039/0.056	0.038/0.060	0.037/0.065	0.051/0.092	0.036/0.049
6	−0.032/0.082	−0.009/0.105	−0.029/0.365*	0.031/0.252*	0.042/0.149*	—	0.024/0.048	0.027/0.051	0.029/0.079	0.026/0.037
7	−0.045/0.005	−0.059/0.050	−0.020/0.240*	−0.004/0.087	−0.019/0.021	−0.019/−0.050	—	0.027/0.056	0.035/0.084	0.026/0.041
8	−0.036/0.348*	−0.040/0.094	−0.005/0.619*	0.020/0.286*	−0.048/0.354*	0.016/0.140	−0.031/0.126	—	0.037/0.087	0.028/0.045
9	−0.015/0.559*	0.046/0.329*	−0.023/0.778*	0.076/0.270	0.106*/0.430*	−0.031/0.475*	0.012/0.383*	0.058/0.517*	—	0.038/0.073
10	0.006/0.150*	0.025/0.155	0.039/0.371*	−0.069/0.233*	−0.003/0.194*	0.043/0.085	0.001/0.034	0.020/0.092	0.086*/0.468*	—

Subpopulations: 1 – El Caballo, 2 – Tozal del Cartujo, 3 – Pico Veleta, 4 – Corral del Veleta/Collado de las Yeguas, 5 – Cerro de los Machos, 6 – Cerro de San Juan/Loma del Mulhacén, 7 – Pico Mulhacén, 8 – Pico del Globo, 9 – Puntal de Vacares, 10 – Pico del Cuervo/Puntal de los Cuartos.
*Significance code: F_{ST} with P-value ≤ 0.05 based on 20 000 permutations.

(Table 3), indicating insignificant population differentiation.

The MRBAYES analysis of AGT1 sequences (Fig. 3A) recovered a well-resolved tree with eight differentiated lineages. Four of these lineages (1, 2, 3 and 8) were widespread, whereas the remaining four (4, 5, 6 and 7) were restricted to the central subpopulations. The ptDNA haplotype network obtained with TCS (Fig. 3B) revealed 15 haplotypes and two missing haplotypes for *L. glacialis* samples. Other two missing haplotypes separated *L. verticillata* and *L. glacialis* samples. The spatial representation of AGT1 lineages and ptDNA haplotypes illustrated the lack of geographical structure as most lineages and haplotypes were distributed throughout the species range.

The MIGRATE-n model testing strongly supported a single panmictic population against the population groups defined by SAMOVA (Model probability$_{m1}$ = ~1; Table 4).

Population size history

The BSP and EBSP showed congruent results (Fig. 4). Neither of the two analyses re-covered constant population size through time for *L. glacialis*. This was further validated by the posterior median for the parameter PopulationSizeChanges in the EBSP which was 2 with 95% HPD 1-3. The BSP showed more resolution in the plot as more coalescent intervals (population size changes) were considered (10). The demographic history shows a constant size until a population decline started in the late Holocene (~1500 ya; Fig. 4), reaching the lowest values between 500 and 200 ya. This decline was followed by a rapid increment in the population size coinciding with the last phases of the Little Ice Age period in Sierra Nevada (N_e ~3500/~30 000 and ~3000/~15 000 in BSP and EBSP, respectively, min/max of median values).

Distribution modelling

Current conditions. The modelled current environmental suitability was markedly similar to the currently known distribution of *L. glacialis*, which mostly overlaps with the alpine vegetation belt of Sierra Nevada as described by Fernández Calzado & Molero Mesa (2011). This occurred in the two analyses: (i) using WorldClim database layers (Fig. 5E) and (ii) using SNOGC database layers (Fig. 6E). Analysis using SNOGC data without the edaphology layer retrieved similar results, but included one more suitable area (results not shown). We failed to find *L. glacialis* individuals in this area during field trips.

The environmental variable that contributed most to explain the MAXENT model under WorldClim database

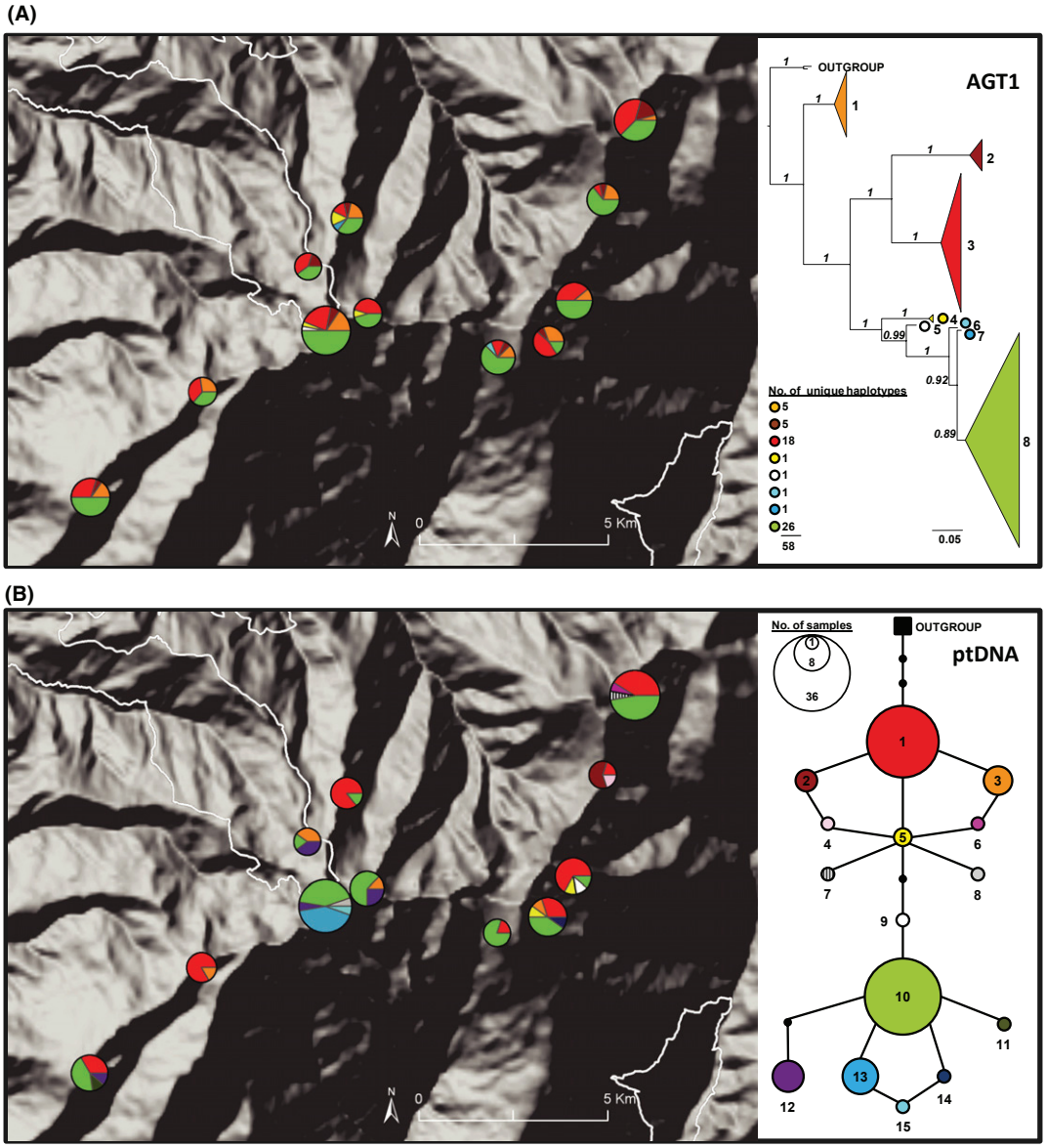

Fig. 3 (A) MrBayes 50% majority rule consensus tree of AGT1 sequences of *Linaria glacialis* and their geographical distribution. Numbers above branches indicate Bayesian posterior probabilities. Clades are numbered (1–8) and number of haplotypes per clade is indicated in the legend; (B) TCS haplotype network of concatenated ptDNA sequences of *Linaria glacialis*. Haplotypes are numbered. Black square represents the outgroup species (*L. verticillata*); lines represent single mutational steps; black small circles are missing haplotypes. Circle sizes indicate number of samples per haplotype. Haplotypes were obtained considering gaps as fifth character.

layers was the minimum temperature of coldest month (bio6) (95%). The most important environmental variables when used alone were bio4, bio5, bio6, bio13 and bio14, according to the jackknife test. The mean value of the maximum training sensitivity plus specificity logistic threshold was 0.22. The average AUC for the replicate runs was 0.988, which indicated a good fit of the model (see Data S3, Supporting information, for further analysis details). The environmental variable that contributed most to explain the Maxent model under SNOGC database layers was edaphology (73.8%). The most important environmental variable when used alone was the average minimum temperature according to the jackknife test. The mean value of the maximum training sensitivity plus specificity logistic threshold was 0.08. The average AUC for the replicate runs was 0.993, which also indicated a good fit of the model (see Data S4, Supporting information, for further analysis details).

Table 4 Log marginal likelihoods (ln(mL)), log Bayes factors (LBF) and probability of MIGRATE-N population models.

SAMOVA models	Thermodynamic ln (mL)	Bezier ln (mL)	LBF* (Thermodynamic)	LBF* (Bezier)	Model probability** (Thermodynamic/Bezier)
1 population	−8484.65	−4582.21	—	—	~1
2 populations	−9035.39	−5195.65	1101.00	1227.30	~0
9 populations	−12 447.61	−8188.24	7925.92	7212.48	~0
10 populations	−11 804.10	−7976.62	6638.90	4113.80	~0

*LBF = 2(ln mL(model$_1$)–ln mL(model$_2$)); Following Kass & Raftery (1995), LBF values smaller than −2 suggested preference for model 2 while values larger than 2 suggested preference for model 1.

** $$\text{Prob}\,(\text{model}_i) = \frac{mL_{\text{model}_i}}{\sum_j^n mL_{\text{model}_j}}$$

Past conditions. Projection of suitability to past conditions (LIG, LGM and mid-Holocene; Fig. 5) revealed a similar distribution range, except for a larger suitable area at the LIG compared with other periods (but note that the species may not have existed at the LIG, see Fig. S1, Supporting information) and a habitat contraction at present time compared to the mid-Holocene.

Future conditions. All simulated GCC scenarios strongly affected the potential distribution of *L. glacialis*. The increase in mean temperatures (see Fig. S3, Supporting information) was predicted to dramatically decrease the extent of suitable areas (Fig. 6A–D, F). All simulated scenarios displayed the same pattern of local extinction but with differences in pace. Area extinction was predicted to occur in the following order for the four GCC scenarios: (i) El Caballo + Cerro de San Juan (simultaneously), (ii) Loma del Mulhacén + Tozal del Cartujo (simultaneously), (iii) Collado de las Yeguas/Corral del Veleta, (iv) Puntal de Vacares, (v) Pico Veleta, (vi) Cerro de los Machos, (vii) Pico del Cuervo/Puntal de los Cuartos, (viii) Pico del Globo and (ix) Pico Mulhacén (Fig. 6A–D, see also Fig. 1C for subpopulation names). The extinction of the last subpopulation (Pico Mulhacén), and thus the extinction of the species, was predicted to occur at two different times under three GCC scenarios: 2100 (CGCM-a2 and ECHAM-b2) and 2075 (ECHAM-a2). Under the CGCM-b2 scenario, the complete extinction of the last subpopulation was not recorded within the time frame of the analysis.

Population size projections

The N_e projections under the four GCC scenarios gave congruent results for the two loci analysed (AGT1 and ptDNA; Fig. 7 and Fig. S4). All median estimates supported approximately constant population sizes (around 30 000 AGT1 gene copies and 10 000 ptDNA gene copies) during the present century until the extinction in the last location is reached.

Discussion

Past, present and future of Linaria glacialis: implications for the conservation of alpine flora

Throughout its history during the late Quaternary climatic changes, *L. glacialis* may have persisted on the Sierra Nevada summits while experiencing contraction–expansion processes (Fig. 5). However, such processes seem to have been limited, if compared with the severe range contractions of future projections (Fig. 6). In the last millennium, climatic oscillations moderately affected the demographic trends of this species (Fig. 4). A slight population size decline was initiated in the early stages of the last Holocene warm period (Medieval Warm Period, MWP *sensu* Mann *et al.* 2009), which was followed by a recovery in size and a subsequent expansion that coincided with the following cold stage (Little Ice Age, LIA *sensu* Gómez-Ortiz *et al.* 2009; Mann *et al.* 2009; Fig. 4). Our results indicate that *L. glacialis* currently maintains an unexpectedly high degree of genetic diversity given the estimated N_e (~30 000/~15 000, BSP/EBSP median values; see Fig. 4) compared with the presumed census size (~10 000 ind., see Appendix S1). A larger amount of molecular data would be necessary to confirm the unexpectedly high genetic diversity found in *L. glacialis*. Nevertheless, a similar pattern of high genetic diversity in small populations was previously reported in other alpine species and interpreted as successful retention of genetic variation in the current relict population after contraction processes (Ellstrand & Elam 1993; Lutz *et al.* 2000). Specifically, in the upper zones of Sierra Nevada, high genetic diversity in small populations was previously detected in *Gentiana alpina*, *Kernera saxatilis* and *Papaver alpinum* and associated with presumed local demo-

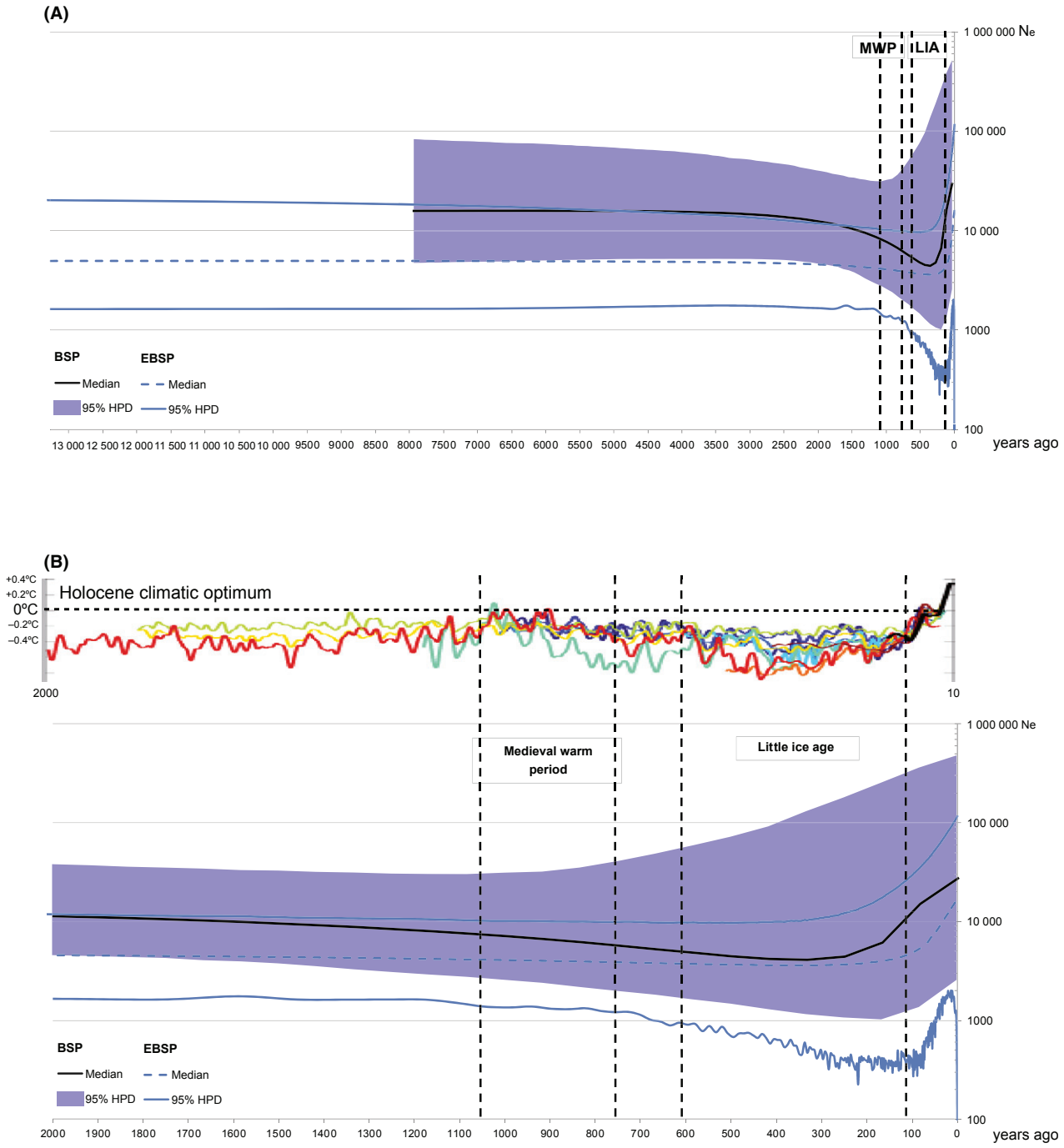

Fig. 4 Bayesian skyline plot (BSP) and extended Bayesian skyline plot (EBSP) representing the historical demographic trends of *Linaria glacialis* in the past 13k years (A) and the past 2k years (B). The *X*-axis represents the time; the *Y*-axis represents effective population size (N_e) considering a generation time of 1 year (N_e values of BSP and EBSP analyses represented gene copies in the population of AGT1 and ptDNA respectively). We also show in B the reconstructed temperature anomalies of the earth during the last 2k years. Temperature graph modified from an original work of Global Warming Art, which is based on previously published data (Jones *et al.* 1998; Bradley *et al.* 1999; Crowley 2000; Briffa *et al.* 2001; Esper *et al.* 2002; Mann & Jones 2003; Huang 2004; Jones & Mann 2004; Moberg *et al.* 2005; Oerlemans 2005) and instrumental data jointly compiled by the Climatic Research Unit (University of East Anglia) and the UK Meteorological Office Hadley Centre; original figure available at http://www.globalwarmingart.com/wiki/File:2000_Year_Temperature_Comparison_png. Limits of Medieval Warm Period (MWP) and Little Ice Age (LIA) according to Mann *et al.* (2009) and Gómez-Ortiz *et al.* (2009).

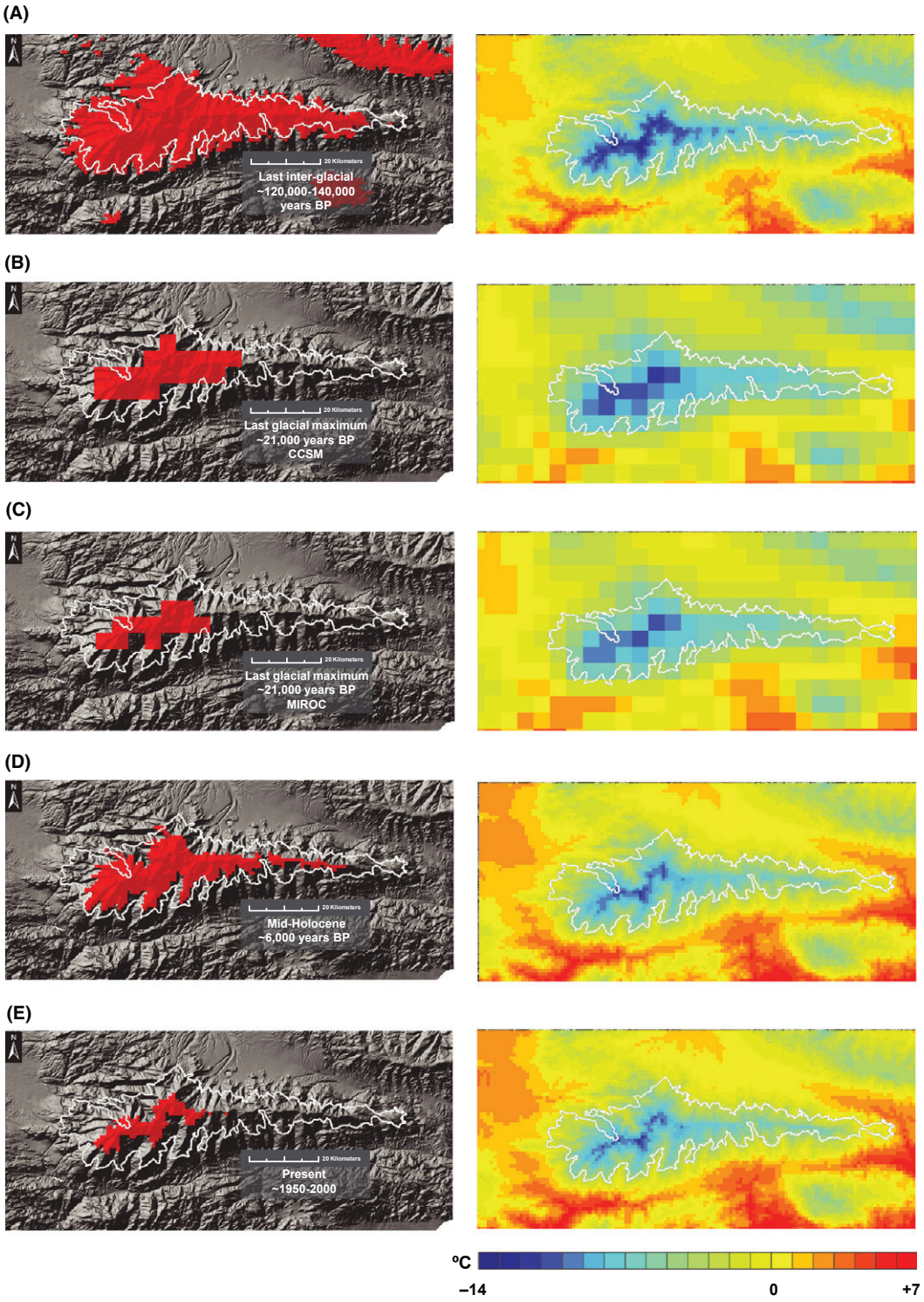

Fig. 5 Results of *Linaria glacialis* distribution modelling based on WorldClim variables and projected to past conditions. The maximum training sensitivity plus specificity logistic threshold was applied to the average output of 10 replicate runs of MAXENT analysis. Projections of the model to: last interglacial (A), last glacial maximum (B, CCSM model; C, MIROC model) and mid-Holocene (D) climatic conditions. (E) Distribution model fitted to current climatic conditions (~1950–2000). Maps on the right represent the minimum temperature of coldest month (bio6) for each period, which is the variable that contributed most to explain the MAXENT model (95%). The white line indicates the limits of the Sierra Nevada National Park.

graphic bottlenecks that were not accompanied by serious losses of diversity (Kropf *et al.* 2006).

Distribution models predicted severe range contractions in the near future (Fig. 6A–D, F), which illustrates the extreme vulnerability of this species to temperature rising. In contrast, projections of future θ_w values indicate that genetic diversity will not diminish at the same pace as the distribution range. That is, future climatic conditions will not lead to a progressive genetic loss, but can lead to species extinction with instantaneous loss of the entire gene pool in a very short time period (Fig. 7). This has important consequences on the way we use genetic diversity as an indicator of threat. The negligible spatial genetic structure of *L. glacialis* (Tables 2–4, Fig. 3) may be responsible for the observed pattern. The genetic diversity of this species is spatially distributed throughout its current range. This could be a distinctive pattern for alpine endemics in Sierra Nevada, in contrast to Alps and Pyrenees, because of the highly restricted range of the alpine vegetation belt. Despite the fact that genetic diversity of alpine plant species seems independent of their distribution in Iberian, Pyrenean or Alpine regions (reviewed in Alarcón *et al.* 2012), genetic differentiation among populations tends to be higher in Alpine plants with larger distributions (see references in Kropf *et al.* 2006). That suggests that the levels of interpopulation genetic differentiation in alpine plants may be scale-dependent, in a way that isolation and differentiation could be favoured at larger scales whereas dispersal and gene flow might be favoured at smaller scales. However, the pattern of high gene flow on the summits of Sierra Nevada may be affected not only by the short geographical distances per se, but also by the exceptionally strong winds, coupled with autecological species traits. In particular, *L. glacialis* produces winged seeds that are

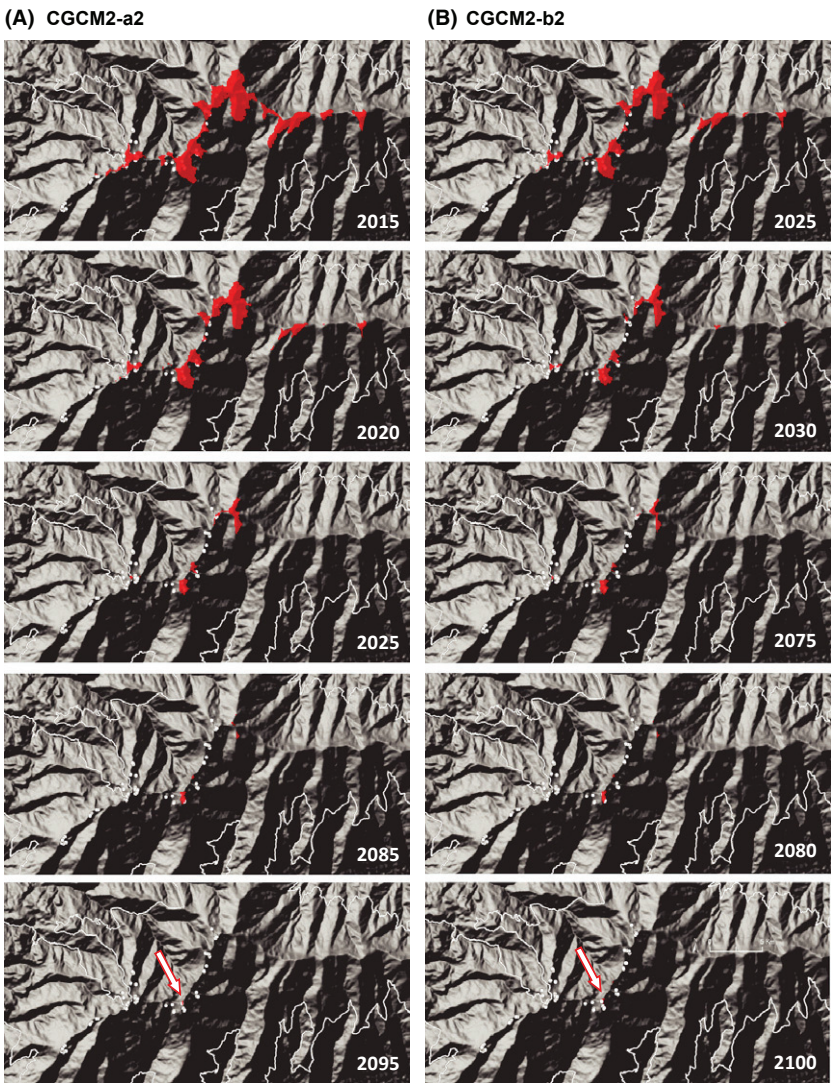

Fig. 6 Results of *Linaria glacialis* distribution modelling based on Sierra Nevada Observatory for Global Change (SNOGC) variables and projected to future global climate change (GCC) scenarios. The maximum training sensitivity plus specificity logistic threshold was applied to the average output of the 10 replicate runs of MAXENT analysis. Projections of the model to the period 2015–2085 under scenarios: CGCM2-a2 (A), CGCM2-b2 (B), ECHAM-a2 (C) and ECHAM-b2 (D). Maps illustrate the successive extinction of the species at sampling sites. (E) Distribution model fitted to current climatic conditions (2010). (F) Projected range contraction in area units (m^2). The white line indicates the limits of the Sierra Nevada National Park, the white dots represent the individuals sampled.

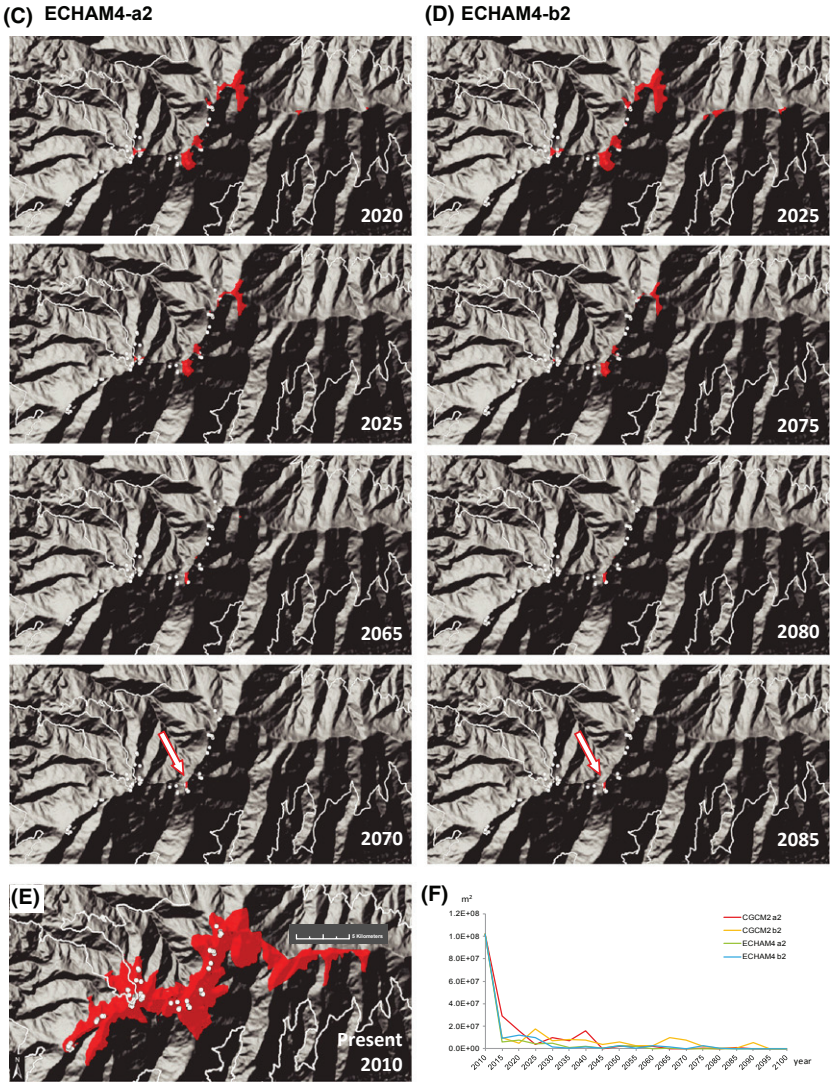

Fig. 6 *Continued.*

probably adapted to wind dispersal (Elisens & Tomb 1983). Also, this species is self-compatible, but has pollen/ovule ratios similar to those of closely related self-incompatible species (J. L. Blanco-Pastor *et al.*, in prep.). Therefore, it is apparent that a mixed mating system has been recently acquired in this endangered species helping promote survival and gene flow in this extreme alpine environment (Gómez 2002; Raffl *et al.* 2007).

Future evolutionary change should not be ignored (Kearney *et al.* 2009; Kuparinen *et al.* 2010; Hoffmann & Sgro 2011). The high genetic diversity found in *L. glacialis* and its spatial distribution lead us to think that resilience of alpine species could be higher than previously thought. However, the short timescale (<100 years) will probably limit adaptive evolution, which would be required for the survival of *L. glacialis* and codistributed taxa. If evolutionary responses are not considered, the projected temperature changes (see Fig. S3, Supporting information) will lead to the disappearance of suitable habitats by the end of the 21st century according to three of the four climate change scenarios analysed (Fig. 6A–D, F). An even more worrying scenario is expected for other alpine species with highly structured genetic diversity and low levels of gene flow because their genetic diversity will be strongly reduced as range contracts. This is of special concern for future conservation programmes given the exceptional level of endemicity and species richness in alpine hotspots.

The effective population size through space and time: a tool for conservation

Given current evidence for a rapid and profound GCC, attention has largely been focused on evaluating and predicting the effects on ecosystems and species. The

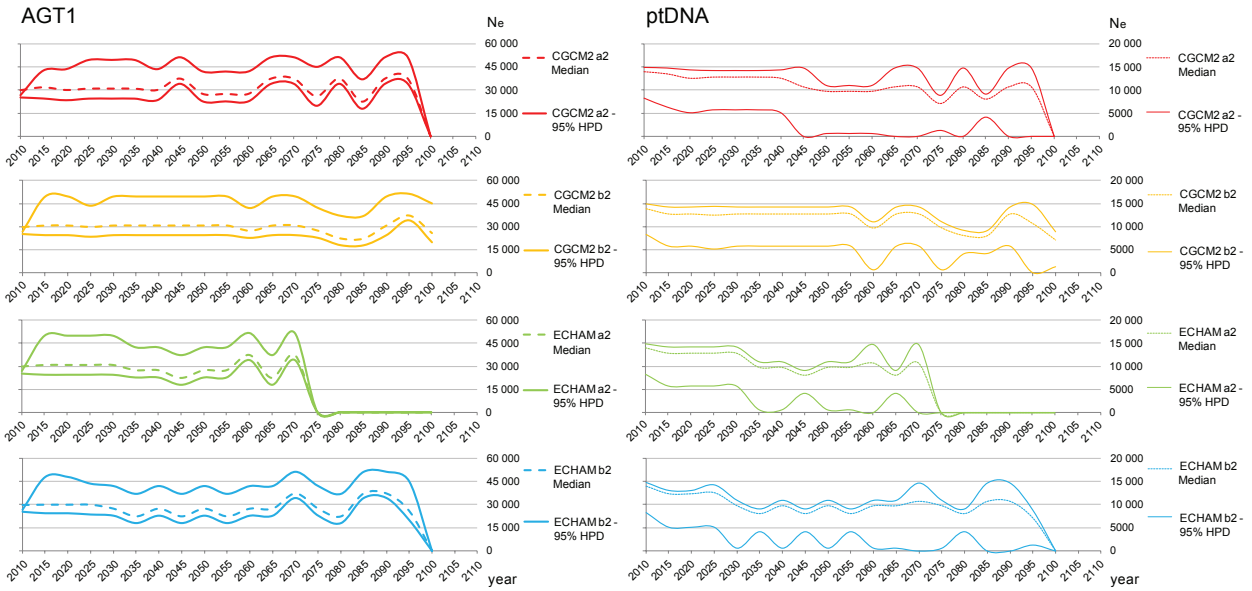

Fig. 7 Projected variation in effective population size of *Linaria glacialis* (N_e) between the years 2015 and 2100 (including parameter uncertainty) under the four projected climate change scenarios. N_e calculations based on sequences of individuals collected at suitable localities according to MAXENT projections. Effective population size estimates were obtained considering a 1-year generation time.

effects on genetic diversity (third component of biodiversity as defined by the United Nations and the UICN; McNeely *et al.* 1990; UNEP 2010) have largely been neglected to date, presumably due to the difficulties to evaluate them.

Historical factors that determined past genetic diversity dynamics have been well addressed, but few studies have explored events over human timescales (hundreds of years) (but see Hoffman *et al.* 2011). A key obstacle when inferring recent historical abundance is the low information content of the most recent coalescent intervals, which prevents the detection of very recent demographic changes (Ho & Shapiro 2011). However, the high mutation rate and variable characters observed in the plastid and particularly in the nuclear sequences of *L. glacialis* (0.57 s/s/Ma and 2.89 s/s/Ma, respectively; EBSP median values, results not shown) allowed us to infer accurate recent demographic reconstructions. To date, few studies have focused on the projected effects of GCC on genetic diversity (but see Espíndola *et al.* 2012; Jay *et al.* 2012; Pfenninger *et al.* 2012). The present study developed a novel approach that can be extended to other endangered species, which helps to link past and future demographic inferences based on the effective population size. In particular, estimates of future N_e were obtained using a combination of analyses that involved the following steps: (i) determining the future habitat suitability for the species using SDMs (e.g. MAXENT), (ii) estimating the population genetic diversity parameter θ_w in the projected suitable habitat (here, we used MIGRATE-n), (iii) estimating the mutation rates of loci (μ) by means of dating analyses (e.g. *BEAST and Bayesian skyline plots) and (iv) applying $N_e = \theta_w/x\mu$ independently for each panmictic unit, to determine the N_e at different stages of the projected habitat contraction.

In the present study, past demographic estimates were based on the coalescent, which is considered to be an efficient framework for evaluating the genetic diversity over time (Crandall *et al.* 1999; Drummond *et al.* 2005; Heled & Drummond 2008; Ho & Shapiro 2011). By contrast, future demographic estimates were obtained by calculating the parameter theta (θ_w) in surviving sampling sites at discrete time points (5-year intervals during the period 2010–2100). Some noteworthy aspects need to be considered when comparing past N_e values with future predictions. First, future N_e values produced using MIGRATE-n do not represent point-in-time estimates, but instead represent the historical mean values along the genealogy of the samples used. Second, the allele frequencies obtained in current localities will not coincide with future allele frequencies because a static equilibrium (absence of mutation, migration or drift) might not be a realistic scenario. Third, the evolutionary potential of the species (niche evolution) was not considered. Therefore, N_e values derived using this method must be interpreted with caution. However, it is important to note that our

method follows the precautionary principle because processes such as migration or niche evolution might help counter extinction. Overall, this approach may be highly valuable to detect risks of genetic diversity loss under GCC and to set priorities during conservation planning.

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References

Alarcón M, Vargas P, Sáez L, Molero J, Aldasoro JJ (2012) Genetic diversity of mountain plants: two migration episodes of Mediterranean *Erodium* (Geraniaceae). *Molecular Phylogenetics and Evolution*, **63**, 866–876.

Beerli P (2012a) *Migrate Documentation*. Version 3.2.1. Department of Scientific Computing. Florida State University. Available from: http://popgen.sc.fsu.edu/migratedoc.pdf.

Beerli P (2012b) Tutorial: comparison of gene flow models using Bayes Factors. http://popgen.sc.fsu.edu/Migrate/Tutorials/Entries/2010/7/12_Day_of_longboarding.html. Accessed December 2012.

Beerli P, Palczewski M (2010) Unified framework to evaluate panmixia and migration direction among multiple sampling locations. *Genetics*, **185**, 313–326.

Benito B, Martínez-Ortega MM, Muñoz L, Lorite J, Peñas J (2009) Assessing extinction-risk of endangered plants using species distribution models: a case study of habitat depletion caused by the spread of greenhouses. *Biodiversity and Conservation*, **18**, 2509–2520.

Benito B, Lorite J, Peñas J (2011) Simulating potential effects of climatic warming on altitudinal patterns of key species in Mediterranean-alpine ecosystems. *Climatic Change*, **108**, 471–483.

Bisconti R, Canestrelli D, Colangelo P, Nascetti G (2011) Multiple lines of evidence for demographic and range expansion of a temperate species (*Hyla sarda*) during the last glaciation. *Molecular Ecology*, **20**, 5313–5327.

Blanco-Pastor JL, Vargas P, Pfeil BE (2012) Coalescent simulations reveal hybridization and incomplete lineage sorting in Mediterranean *Linaria*. *PLoS One*, **7**, e39089.

Braconnot P, Otto-Bliesner B, Harrison S *et al*. (2007) Results of PMIP2 coupled simulations of the Mid-Holocene and last glacial maximum – Part 1: experiments and large-scale features. *Climate of the Past*, **3**, 261–277.

Bradley RS, Mann M, Hughes MK (1999) Northern hemisphere temperatures during the past millennium: inferences, uncertainties, and limitations. *Geophysical Research Letters*, **26**, 759–762.

Briffa KR, Osborn TJ, Schweingruber FH *et al*. (2001) Low-frequency temperature variations from a northern tree ring density network. *Journal of Geophysical Research*, **106**, 2929–2941.

Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology*, **9**, 1657–1660.

CMA (2005) Mapa de suelos de Andalucía: unidades edáficas. 1:400000. Consejería de Medio Ambiente (CMA), Junta de Andalucía. http://www.juntadeandalucia.es. Publishers: Consejería de Agricultura, Junta de Andalucía and CSIC (1989)/Landsat T.M.

Collins WD, Bitz CM, Blackmon ML *et al*. (2006) The community climate system model version 3 (CCSM3). *Journal of Climate*, **19**, 22.

Cordellier M, Pfenninger M (2009) Inferring the past to predict the future: climate modelling predictions and phylogeography for the freshwater gastropod *Radix balthica* (Pulmonata, Basommatophora). *Molecular Ecology*, **18**, 534–544.

Crandall KA, Posada D, Vasco D (1999) Effective population sizes: missing measures and missing concepts. *Animal Conservation*, **2**, 317–319.

Crowley TJ (2000) Causes of climate change over the past 1000 years. *Science*, **289**, 270–277.

Dépraz A, Cordellier M, Hausser J, Pfenninger M (2008) Postglacial recolonization at a snail's pace (*Trochulus villosus*): confronting competing refugia hypotheses using model selection. *Molecular Ecology*, **17**, 2449–2462.

Diaz H, Grosjean M, Graumlich L (2003) Climate variability and change in high elevation regions: past, present and future. *Climatic Change*, **59**, 1–4.

Drummond AJ, Rambaut A, Shapiro B, Pybus OG (2005) Bayesian coalescent inference of past population dynamics from molecular sequences. *Molecular Biology and Evolution*, **22**, 1185–1192.

Drummond AJ, Suchard MA, Xie D, Rambaut A (2012) Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology and Evolution*, **29**, 1969–1973.

Dupanloup I, Schneider S, Excoffier L (2002) A simulated annealing approach to define the genetic structure of populations. *Molecular Ecology*, **11**, 2571–2581.

Elisens WJ, Tomb AS (1983) Seed morphology in New World Antirrhineae (Scrophulariaceae): systematic and phylogenetic implications. *Plant Systematics and Evolution*, **142**, 23–47.

Elith J, Graham CH, Anderson RP *et al*. (2006) Novel methods improve prediction of species' distributions from occurrence data. *Ecography*, **29**, 129–151.

Ellstrand NC, Elam DR (1993) Population genetic consequences of small population size: implications for plant conservation. *Annual Review of Ecology and Systematics*, **24**, 217–242.

Engler R, Randin CF, Thuiller W *et al*. (2011) 21st century climate change threatens mountain flora unequally across Europe. *Global Change Biology*, **17**, 2330–2341.

Esper J, Cook ER, Schweingruber FH (2002) Low-frequency signals in long tree-ring chronologies for reconstructing past temperature variability. *Science*, **295**, 2250–2253.

Espíndola A, Pellissier L, Maiorano L *et al*. (2012) Predicting present and future intra-specific genetic structure through

niche hindcasting across 24 millennia. *Ecology Letters*, **15**, 649–657.

Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources*, **10**, 564–567.

Fernández Calzado MR, Molero Mesa J (2011) The cartography of vegetation in the cryoromediterranean belt of Sierra Nevada: a tool for biodiversity conservation. *Lazaroa*, **32**, 101–115.

Fernández-Mazuecos M, Vargas P (2013) Congruence between distribution modelling and phylogeographical analyses reveals Quaternary survival of a toadflax species (*Linaria elegans*) in oceanic climate areas of a mountain ring range. *New Phytologist*, **198**, 1274–1289.

Fernández-Mazuecos M, Blanco-Pastor JL, Vargas P (2013) A phylogeny of toadflaxes (*Linaria* Mill.) based on nuclear internal transcribed spacer sequences: systematic and evolutionary consequences. *International Journal of Plant Sciences*, **174**, 234–249.

Fu YX, Li WH (1993) Statistical tests of neutrality of mutations. *Genetics*, **133**, 693–709.

Gómez JM (2002) Self-pollination in *Euphrasia willkommii* Freyn (Scrophulariaceae), an endemic species from the alpine of the Sierra Nevada (Spain). *Plant Systematics and Evolution*, **232**, 63–71.

Gomez-Campo C, Bermudez-De-Castro L, Cagiga MJ, Sánchez-Yelamo MD (1984) Endemism in the Iberian Peninsula and Balearic Islands. *Webbia*, **38**, 709–714.

Gómez-Ortiz A, Palacios D, Schulte L, Salvador-Franch F, Plana-CastellvÍ JA (2009) Evidences from historical documents of landscape evolution after Little Ice Age of a Mediterranean high mountain area, Sierra Nevada, Spain (eighteenth to twentieth centuries). *Geografiska Annaler: Series A, Physical Geography*, **91**, 279–289.

Gottfried M, Pauli H, Futschik A *et al.* (2012) Continent-wide response of mountain vegetation to climate change. *Nature Climate Change*, **2**, 111–115.

Guisan A, Thuiller W (2005) Predicting species distribution: offering more than simple habitat models. *Ecology Letters*, **8**, 993–1009.

Hasumi H, Emori S (2004) *K-1 Coupled Model (MIROC) Description (K-1 Technical Report 1)*. Center for Climate System Research, University of Tokyo, Tokyo.

Heled J, Drummond A (2008) Bayesian inference of population size history from multiple loci. *BMC Evolutionary Biology*, **8**, 289.

Hijmans RJ, Cameron SE, Parra JL, Jones PG, Jarvis A (2005) Very high resolution interpolated climate surfaces for global land areas. *International Journal of Climatology*, **25**, 1965–1978.

Ho SYW, Shapiro B (2011) Skyline-plot methods for estimating demographic history from nucleotide sequences. *Molecular Ecology Resources*, **11**, 423–434.

Hoffman JI, Grant SM, Forcada J, Phillips CD (2011) Bayesian inference of a historical bottleneck in a heavily exploited marine mammal. *Molecular Ecology*, **20**, 3989–4008.

Hoffmann AA, Sgro CM (2011) Climate change and evolutionary adaptation. *Nature*, **470**, 479–485.

Huang S (2004) Merging information from different resources for new insights into climate change in the past and future. *Geophysical Research Letters*, **31**, L13205.

Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogeny. *Bioinformatics*, **17**, 754–755.

Jay F, Manel S, Alvarez N *et al.* (2012) Forecasting changes in population genetic structure of alpine plants in response to global warming. *Molecular Ecology*, **21**, 2354–2368.

Jaynes ET (1957) Information theory and statistical mechanics. *Physical Review*, **106**, 620–630.

Jiménez-Valverde A, Lobo JM (2007) Threshold criteria for conversion of probability of species presence to either-or presence-absence. *Acta Oecologica*, **31**, 361–369.

Jones PD, Mann ME (2004) Climate over past millennia. *Reviews of Geophysics*, **42**, RG2002.

Jones PD, Briffa KR, Barnett TP, Tett SFB (1998) High-resolution palaeoclimatic records for the last millennium: interpretation, integration and comparison with General Circulation Model control-run temperatures. *The Holocene*, **8**, 455–471.

Jost L (2008) GST and its relatives do not measure differentiation. *Molecular Ecology*, **17**, 4015–4026.

Kass RE, Raftery AE (1995) Bayes factors. *Journal of the American Statistical Association*, **90**, 773–795.

Kearney M, Porter WP, Williams C, Ritchie S, Hoffmann AA (2009) Integrating biophysical models and evolutionary theory to predict climatic impacts on species' ranges: the dengue mosquito *Aedes aegypti* in Australia. *Functional Ecology*, **23**, 528–538.

Kropf M, Comes HP, Kadereit JW (2006) Long-distance dispersal vs vicariance: the origin and genetic diversity of alpine plants in the Spanish Sierra Nevada. *New Phytologist*, **172**, 169–184.

Kuparinen A, Savolainen O, Schurr FM (2010) Increased mortality can promote evolutionary adaptation of forest trees to climate change. *Forest Ecology and Management*, **259**, 1003–1008.

Lartillot N, Philippe H (2006) Computing Bayes factors using thermodynamic integration. *Systematic Biology*, **55**, 195–207.

Leblois R, Estoup A, Streiff R (2006) Genetics of recent habitat contraction and reduction in population size: does isolation by distance matter? *Molecular Ecology*, **15**, 3601–3615.

Librado P, Rozas J (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, **25**, 1451–1452.

Lutz E, Schneller JJ, Holderegger R (2000) Understanding population history for conservation purposes: population genetics of *Saxifraga aizoides* (Saxifragaceae) in the lowlands and lower mountains north of the Alps. *American Journal of Botany*, **87**, 583–590.

Mann ME, Jones PD (2003) Global surface temperatures over the past two millennia. *Geophysical Research Letters*, **30**, 1820.

Mann ME, Zhang Z, Rutherford S *et al.* (2009) Global signatures and dynamical origins of the Little Ice Age and Medieval Climate Anomaly. *Science*, **326**, 1256–1260.

Martin DP, Lemey P, Lott M *et al.* (2010) RDP3: a flexible and fast computer program for analyzing recombination. *Bioinformatics*, **26**, 2462–2463.

McNeely JA, Miller KR, Reid WV, Mittermeier RA, Werner TB (1990) *Conserving the World's Biological Diversity*. IUCN, WRI, WWF-US, WorldBank, Conservation International, Gland, Switzerland/Washington, DC.

Médail F, Quézel P (1997) Hot-spots analysis for conservation of plant biodiversity in the Mediterranean basin. *Annals of the Missouri Botanical Garden*, **84**, 112–127.

Médail F, Quézel P (1999) Biodiversity hotspots in the Mediterranean Basin: setting global conservation priorities. *Conservation Biology*, **13**, 1510–1513.

Meirmans PG, Hedrick PW (2011) Assessing population structure: FST and related measures. *Molecular Ecology Resources*, **11**, 5–18.

Meirmans PG, Van Tienderen PH (2004) GENOTYPE and GENODIVE: two programs for the analysis of genetic diversity of asexual organisms. *Molecular Ecology Notes*, **4**, 792–794.

Moberg A, Sonechkin DM, Holmgren K, Datsenko NM, Karlen W (2005) Highly variable Northern Hemisphere temperatures reconstructed from low- and high-resolution proxy data. *Nature*, **433**, 613–617.

Nei M (1973) Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences*, **70**, 3321–3323.

Nei M (1987) *Molecular Evolutionary Genetics*. Columbia University Press, New York.

Nogués-Bravo D (2009) Predicting the past distribution of species climatic niches. *Global Ecology and Biogeography*, **18**, 521–531.

Oerlemans J (2005) Extracting a climate signal from 169 glacier records. *Science*, **308**, 675–677.

Otto-Bliesner BL, Marshall SJ, Overpeck JT *et al.* (2006) Simulating Arctic climate warmth and icefield retreat in the last interglaciation. *Science*, **311**, 1751–1753.

Parmesan C (2006) Ecological and evolutionary responses to recent climate change. *Annual Review of Ecology, Evolution, and Systematics*, **37**, 637–669.

Pauli H, Gottfried M, Grabherr G (1996) Effects of climate change on mountain ecosystems – upward shifting of alpine plants. *World Resource Review*, **8**, 382–390.

Pauli H, Gottfried M, Hohenwallner D, Reiter K, Grabherr G (2004) *The GLORIA Field Manual–Multi-Summit Approach*. Office for Official Publications of the European Communities, Brussels.

Pauli H, Gottfried M, Dullinger S *et al.* (2012) Recent plant diversity changes on Europe's mountain summits. *Science*, **336**, 353–355.

Pauls SU, Nowak C, Bálint M, Pfenninger M (2013) The impact of global climate change on genetic diversity within populations and species. *Molecular Ecology*, **22**, 925–946.

Pfenninger M, Bálint M, Pauls SU (2012) Methodological framework for projecting the potential loss of intraspecific genetic diversity due to global climate change. *BMC Evolutionary Biology*, **12**, 224.

Phillips SJ, Anderson RP, Schapire RE (2006) Maximum entropy modeling of species geographic distributions. *Ecological Modelling*, **190**, 231–259.

Posada D (2008) jModelTest: phylogenetic model averaging. *Molecular Biology and Evolution*, **25**, 1253–1256.

Raffl C, Marcante S, Erschbamer B (2007) The role of spontaneous selfing in the pioneer species *Saxifraga aizoides*. *Flora: Morphology Distribution, Functional Ecology of Plants*, **202**, 128–132.

Rambaut A, Drummond AJ (2009) Tracer v1.5. Available from BEAST Software website: http://tree.bio.ed.ac.uk/software/tracer/. Accessed October 2012.

Roeckner E, Arpe K, Bengtsson L *et al.* (1992) Simulation of the present-day climate with the ECHAM model: impact of model physics and resolution. Report 93. Max-Planck-Institut für Meteorologie, Germany.

Rosenzweig C, Karoly D, Vicarelli M *et al.* (2008) Attributing physical and biological impacts to anthropogenic climate change. *Nature*, **453**, 353–357.

Scheen A-C, Pfeil BE, Petri A *et al.* (2011) Use of allele-specific sequencing primers is an efficient alternative to PCR subcloning of low-copy nuclear genes. *Molecular Ecology Resources*, **12**, 128–135.

Scoble J, Lowe AJ (2010) A case for incorporating phylogeography and landscape genetics into species distribution modelling approaches to improve climate adaptation and conservation planning. *Diversity and Distributions*, **16**, 343–353.

Stephens M, Donnelly P (2003) A comparison of Bayesian methods for haplotype reconstruction from population genotype data. *The American Journal of Human Genetics*, **73**, 1162–1169.

Stephens M, Smith NJ, Donnelly P (2001) A new statistical method for haplotype reconstruction from population data. *The American Journal of Human Genetics*, **68**, 978–989.

Tajima F (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics*, **123**, 585–595.

Thomas CD, Cameron A, Green RE *et al.* (2004) Extinction risk from climate change. *Nature*, **427**, 145–148.

Thuiller W, Lavorel S, Araújo MB, Sykes MT, Prentice IC (2005) Climate change threats to plant diversity in Europe. *Proceedings of the National Academy of Sciences of the United States of America*, **102**, 8245–8250.

UNEP (2010) COP 10 Decision X/2. Strategic Plan for Biodiversity 2011–2020. In: *Conference of the parties to the convention on biological diversity*, Nagoya, Japan, 18–29 October.

Walther G-R, Beißner S, Burga CA (2005) Trends in the upward shift of alpine plants. *Journal of Vegetation Science*, **16**, 541–548.

Watterson GA (1975) On the number of segregating sites in genetical models without recombination. *Theoretical Population Biology*, **7**, 256–276.

Wright S (1943) Isolation by distance. *Genetics*, **28**, 114.

Yang Z (2006) *Computational Molecular Evolution*. Oxford University Press, Oxford.

J.L.B.-P. designed research, performed lab work, analyzed data, wrote the paper. M.F.-M. analyzed data and P.V. designed research. All authors contributed to fieldwork, data interpretation and proof reading.

Data accessibility

DNA sequences: GenBank Accessions KC625597–KC625997.

Sampling details for each individual: sampling site, collection number, GPS coordinates and the GenBank Accession number in the online Supplementary Table 2.

DNA sequence assembly used in MIGRATE-n analyses uploaded as online supplementary data.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Characteristics of the DNA regions sequenced.

Table S2 List of accessions with localities, collection numbers, GenBank accession numbers and GPS coordinates.

Table S3 Allele-specific primers (ASP) designed for the amplification of AGT1 haplotypes of the *L. glacialis* data set.

Fig. S1 Species tree of Linaria sect. Supinae subsect. Supinae *sensu* Blanco-Pastor (2012), with *L. arvensis* and *L. saxatilis* as outgroup.

Fig. S2 AGT1 dated tree of *L. glacialis* and substitution rates (right plot) as obtained from the Bayesian skyline plot analysis. AGT1 substitution rates of the sect. *Supinae* and subsect. *Supinae* datasets are also shown (left plot).

Fig. S3 (A–E) Maps showing the variation of average minimum annual temperatures in Sierra Nevada for the period 2010–2100 under the four projected climate change scenarios (A) CGCM2-a2, (B) CGCM2-b2, (C) ECHAM4-a2, (D) ECHAM4-b2.

Fig. S4 Joint representation of the BSP and EBSP analyses for past demographic trends (past 500 years) and future demographic projections (median values of N_e projections for the next 90 years as shown in Fig. 7).

Data S1 (a) Results of MIGRATE-n analyses for model comparison and (b) sequence alignment input file.

Data S2 (a) Results of MIGRATE-n analyses for future projections and (b–j) sequence alignment input files.

Data S3 Results of MAXENT analysis under WorldClim variables.

Data S4 Results of MAXENT analysis under Sierra Nevada Observatory for Global Change (SNOGC) variables.

Appendix S1 Supplementary materials, methods and results.

Supporting Information from Blanco-Pastor *et al.* 2013, "Past and future demographic dynamics of alpine species: limited genetic consequences despite dramatic range contraction in a plant from the Spanish Sierra Nevada"

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Supplementary table 1. Characteristics of the DNA regions sequenced.

	Species-level dataset		***L. glacialis* dataset**			
	ITS (ITS1-5.8S-ITS2)	**AGT1 intron**	**AGT1 exon**	**AGT1 intron**	**rpl32-trnL^{UAG}**	**3′rps16-trnK^{UUU}**
Description	*Internal transcribed spacers 1 and 2 and 5.8S ribosomal RNA*	*Partial intron of the alanine glyoxylate aminotransferase (photorespiratory enzyme)*	*Partial exon of the alanine glyoxylate aminotransferase (photorespiratory enzyme)*	*Partial intron of the alanine glyoxylate aminotransferase (photorespiratory enzyme)*	*Intergenic spacer of SSC chloroplast region*	*Intergenic spacer of LSC chloroplast region*
References	White (1990)	Liepman & Olsen (2001)	Liepman & Olsen (2001)	Liepman & Olsen (2001)	Shaw *et al.* (2007)	Shaw *et al.* (2007)
No. of newly generated sequences used (haplotypes)	28	28	185	183	100	102
No. of previously published sequences used (haplotypes)	8	5	0	2	2	0
Total number of sequences in the alignment (haplotypes)	36	33	185	185	102	102
Aligned length (bp)	587	446	255	398	687	517
Ungapped length range	583-586	379-428	253-255	322-360	628-669	507-516
% Identical sites	84.7%	43.7%	92.9%	49.5%	89.1%	96.7%
% Pairwise identity	96.7%	88.2%	98.5%	89.0%	99.2%	99.3%
Variable characters	72	143	16	93	10	10
Parsimony-informative characters	60	124	14	82	2	5
Mean % G+C content	57.8%	33.4%	46.7%	30.7%	24.7%	28.6%
Substitution model	GTR+I+G	GTR+G	GTR+I+G	GTR+G	GTR+G	HKY+I+G

Supplementary table 2. List of accessions included in the present study with localities, collection numbers and Genbank accession numbers.

Taxon	Sampling Site	Collection number	ITS	*AGT1*	*rpl32-trnL*^{UAG}	*rps16-trnK*^{UUU}
Species-level dataset						
L. aeruginea (Gouan) Cav. ssp. *aeruginea*	Spain: Granada. Sierra Nevada. Pradollano	51JB09	JQ814486	JQ814558	-	-
L. aeruginea ssp. *nevadensis* (Boiss.) D.A. Sutton	Spain: Granada. Sierra Nevada. Pico del Veleta	44JB09	JQ814487	JQ814559	-	-
L. almijarensis Campo & Amo	Spain: Córdoba. Cabra.	36JB10	JQ814492	JQ814564	-	-
L. amoi Campo ex Amo	Spain: Sierra Tejeda. Canillas de Aceituno	37JB09	KC625802 KC625803	KC625806 KC625807	-	-
L. anticaria Boiss. & Reut.	Spain: Málaga. El Torcal de Antequera	33JB09	JQ814491	JQ814563	-	-
L. arvensis (L.) Desf. (1)	France: Córse. Col de Bigorno.	2009.12.169 (RNG) (J. Lambinon)	JQ814493	JQ814565	-	-
L. arvensis (L.) Desf. (2)	Spain: Almería. Uleila del Campo	2009.12.165 (RNG) (S.L. Jury & R.N. Carter)	JN663636	JQ814566	-	-
L. depauperata ssp. *hegelmaieri* (Lange) De la Torre, Alcaraz & M.B. Crespo	Spain: Alicante. Petrel	6JB10	KC625799	KC625809	-	-
L. lilacina Lange.	Spain: Jaén. Otiñar	16JB12_1	JX481156	KC625812	-	-
L. platycalyx Boiss.	Spain: Cádiz. Zahara de la Sierra	5SMB08 (S. Martín Bravo)	JQ814520	JQ814592	-	-
L. polygalifolia ssp. lamarckii (Rouy) D.A. Sutton. (1)	Portugal: Algarve. Monte Gordo	33JB10	JQ814522	JQ814594	-	-
L. polygalifolia ssp. lamarckii (Rouy) D.A. Sutton. (2)	Spain: Huelva. Isla Canela.	19JB09	JQ814521	JQ814593	-	-
L. saxatilis (L.) Chaz.	Spain: Ávila. Hoyos del Espino	94PV09 (P.Vargas)	JQ814526	JQ814597	-	-
L. supina (L.) Chaz. *ssp. supina*	France: Gorges de l'Hérault	2009.12.131 (RNG) (J. Lambinon)	JQ814530	JQ814601	-	-
L. tristis (L.) Mill. ssp. *tristis*	Spain: Cádiz. Alcalá de los Gazules	105PJM04 (P. Jiménez Mejías)	KC625800 KC625801	KC625808	-	-
L. verticillata Boiss. (1)	Spain: Granada. Sierra	2009.12.53 (RNG) (J.M.	KC625804	KC625996/	KC625798	KC625697

	Nevada. Las Sabinas	Losa)	KC625805	KC625810 KC625997/ KC625811				
***L. glacialis* dataset**							**GPS Coordinates (decimal. degrees)**	
							Lat.	**Long.**
L. glacialis Boiss.	Pico Veleta (1)	45JB09_1	-	KC625931 KC625932	KC625761	KC625660	37,0505	-3,3719
L. glacialis Boiss.	Pico Veleta (2)	45JB09_2	-	KC625955 -	-	-	37,0499	-3,3718
L. glacialis Boiss.	Pico Veleta (3)	45JB09_3	-	KC625958 KC625959	KC625776	KC625675	37,0495	-3,3714
L. glacialis Boiss.	Pico Veleta (4)	45JB09_4	-	- -	KC625796	KC625695	37,0491	-3,3734
L. glacialis Boiss.	Pico Veleta (5)	45JB09_5	-	KC625898 KC625899	KC625743	KC625642	37,0487	-3,3718
L. glacialis Boiss.	Pico Veleta (6)	45JB09_6	-	KC625907 KC625908	KC625748	KC625647	37,0483	-3,3718
L. glacialis Boiss.	Pico Veleta (7)	45JB09_10	-	- -	KC625779	KC625678	37,0533	-3,3699
L. glacialis Boiss.	Pico Veleta (8)	45JB09_11	-	KC625889 -	KC625738	KC625637	37,0526	-3,3696
L. glacialis Boiss.	Pico Veleta (9)	45JB09_12	-	KC625947 KC625948	KC625771	KC625670	37,0524	-3,3702
L. glacialis Boiss.	Pico Veleta (10)	45JB09_13	-	KC625953 KC625954	KC625774	KC625673	37,0511	-3,3708
L. glacialis Boiss.	Pico Veleta (11)	45JB09_14	-	KC625956 KC625957	KC625775	KC625674	37,0518	-3,3715
L. glacialis Boiss.	Pico Veleta (12)	43JB09_1	JQ814504	KC625890 KC625891	KC625739	KC625638	37,0561	-3,3687
L. glacialis Boiss.	Pico Veleta (13)	43JB09_2	-	KC625949 KC625950	KC625772	KC625671	37,0569	-3,3688
L. glacialis Boiss.	Pico Veleta (14)	43JB09_3	-	KC625827 KC625828	KC625706	KC625605	37,0565	-3,3691
L. glacialis Boiss.	Pico Veleta (15)	43JB09_4	-	KC625833 KC625834	KC625709	KC625608	37,0561	-3,3691
L. glacialis Boiss.	Pico Veleta (16)	43JB09_5	-	KC625843	KC625714	KC625613	37,0556	-3,3691

				KC625844				
L. glacialis Boiss.	Pico Veleta (17)	43JB09_6	-	KC625851 KC625852	KC625718	KC625617	37,0552	-3,3690
L. glacialis Boiss.	Pico Veleta (18)	43JB09_7	-	KC625857 KC625858	KC625721	KC625620	37,0548	-3,3692
L. glacialis Boiss.	Pico Veleta (19)	43JB09_8	-	KC625865 KC625866	KC625725	KC625624	37,0544	-3,3694
L. glacialis Boiss.	Pico Veleta (20)	43JB09_9	-	- -	KC625730	KC625629	37,0539	-3,3696
L. glacialis Boiss.	Collado de las Yeguas (1)	65JB09_1	-	KC625894 KC625895	KC625741	KC625640	37,0579	-3,3750
L. glacialis Boiss.	Collado de las Yeguas (2)	73JB09_1	-	KC625879 KC625880	KC625733	KC625632	37,0627	-3,3763
L. glacialis Boiss.	Collado de las Yeguas (3)	73JB09_2	-	KC625969 KC625970	KC625783	KC625682	37,0623	-3,3762
L. glacialis Boiss.	Pico Mulhacén (1)	167JB10_1	-	KC625892 KC625893	KC625740	KC625639	37,0528	-3,3118
L. glacialis Boiss.	Pico Mulhacén (2)	167JB10_2	-	KC625977 KC625978	KC625786	KC625685	37,0532	-3,3118
L. glacialis Boiss.	Pico Mulhacén (3)	167JB10_3	-	KC625902 KC625903	KC625745	KC625644	37,0493	-3,3105
L. glacialis Boiss.	Pico Mulhacén (4)	167JB10_4	-	KC625983 -	KC625790	KC625689	37,0489	-3,3103
L. glacialis Boiss.	Pico Mulhacén (5)	167JB10_5	-	KC625911 KC625912	KC625750	KC625649	37,0489	-3,3103
L. glacialis Boiss.	Pico Mulhacén (6)	167JB10_6	-	KC625990 KC625991	KC625794	KC625693	37,0469	-3,3092
L. glacialis Boiss.	Pico Mulhacén (7)	167JB10_7	-	KC625919 KC625920	KC625755	KC625654	37,0469	-3,3092
L. glacialis Boiss.	Pico Mulhacén (8)	167JB10_8	-	KC625821 KC625822	KC625699	KC625598	37,0471	-3,3091
L. glacialis Boiss.	Pico Mulhacén (9)	167JB10_9	-	KC625929 KC625930	KC625760	KC625659	37,0471	-3,3091
L. glacialis Boiss.	Pico Mulhacén (10)	167JB10_10	-	KC625825 KC625826	KC625703	KC625602	37,0476	-3,3088
L. glacialis Boiss.	Loma del Mulhacén (1)	54JB09_1	-	KC625935 KC625936	KC625763	KC625662	37,0512	-3,3291

L. glacialis Boiss.	Loma del Mulhacén (2)	54JB09_2	-	KC625992 KC625993	KC625795	KC625694	37,0522	-3,3219
L. glacialis Boiss.	Loma del Mulhacén (3)	54JB09_3	-	KC625815 KC625816	KC625700	KC625599	37,0470	-3,3202
L. glacialis Boiss.	Loma del Mulhacén (4)	54JB09_4	-	KC625951 KC625952	KC625773	KC625672	37,0465	-3,3204
L. glacialis Boiss.	Loma del Mulhacén (5)	54JB09_5	-	KC625943 KC625944	KC625767	KC625666	37,0459	-3,3205
L. glacialis Boiss.	Cerro de los Machos (1)	79JB09_1	-	KC625904 -	KC625746	KC625645	37,0595	-3,3624
L. glacialis Boiss.	Cerro de los Machos (2)	79JB09_2	-	KC625873 KC625874	KC625729	KC625628	37,0583	-3,3613
L. glacialis Boiss.	Cerro de los Machos (3)	79JB09_3	-	KC625881 KC625882	KC625734	KC625633	37,0593	-3,3621
L. glacialis Boiss.	Cerro de los Machos (4)	80JB09_3	-	- -	KC625788	KC625687	37,0577	-3,3619
L. glacialis Boiss.	Cerro de los Machos (5)	81JB09_1	-	KC625986 KC625987	KC625792	KC625691	37,0541	-3,3619
L. glacialis Boiss.	Cerro de los Machos (6)	81JB09_2	-	KC625994 KC625995	KC625797	KC625696	37,0541	-3,3618
L. glacialis Boiss.	Cerro de los Machos (7)	81JB09_3	-	KC625819 KC625820	KC625702	KC625601	37,0552	-3,3604
L. glacialis Boiss.	Cerro de los Machos (8)	81JB09_4	-	- -	KC625705	KC625604	37,0571	-3,3599
L. glacialis Boiss.	Corral del Veleta (1)	80JB09_1	-	KC625965 KC625966	KC625781	KC625680	37,0707	-3,3672
L. glacialis Boiss.	Corral del Veleta (2)	80JB09_2	-	KC625973 KC625974	KC625784	KC625683	37,0698	-3,3617
L. glacialis Boiss.	Tozal del Cartujo (1)	69JB09_1	-	KC625941 KC625942	KC625766	KC625665	37,0398	-3,4021
L. glacialis Boiss.	Tozal del Cartujo (2)	69JB09_2	-	KC625960 -	KC625777	KC625676	37,0397	-3,4039
L. glacialis Boiss.	Tozal del Cartujo (3)	69JB09_3	-	KC625963 KC625964	KC625780	KC625679	37,0370	-3,4091
L. glacialis Boiss.	Tozal del Cartujo (4)	69JB09_4	-	KC625933 KC625934	KC625762	KC625661	37,0366	-3,4094
L. glacialis Boiss.	Tozal del Cartujo (5)	69JB09_5	-	KC625939	KC625765	KC625664	37,0356	-3,4101

				KC625940				
L. glacialis Boiss.	Tozal del Cartujo (6)	69JB09_6	-	KC625896 KC625897	KC625742	KC625641	37,0348	-3,4107
L. glacialis Boiss.	El Caballo (1)	70JB09_1	JQ814505	KC625921 KC625922	KC625756	KC625655	37,0163	-3,4380
L. glacialis Boiss.	El Caballo (2)	70JB09_2	-	KC625971 KC625972	-	-	37,0152	-3,4394
L. glacialis Boiss.	El Caballo (3)	70JB09_3	-	KC625979 KC625980	KC625787	KC625686	37,0115	-3,4370
L. glacialis Boiss.	El Caballo (4)	70JB09_4	-	KC625859 KC625860	KC625722	KC625621	37,0104	-3,4392
L. glacialis Boiss.	El Caballo (5)	70JB09_5	-	KC625867 KC625868	KC625726	KC625625	37,0111	-3,4410
L. glacialis Boiss.	El Caballo (6)	70JB09_6	-	KC625875 KC625876	KC625731	KC625630	37,0111	-3,4410
L. glacialis Boiss.	El Caballo (7)	70JB09_7	-	KC625883 KC625884	KC625735	KC625634	37,0127	-3,4404
L. glacialis Boiss.	El Caballo (8)	70JB09_8	-	KC625887 KC625888	KC625737	KC625636	37,0130	-3,4411
L. glacialis Boiss.	El Caballo (9)	70JB09_9	-	KC625900 KC625901	KC625744	KC625643	37,0135	-3,4407
L. glacialis Boiss.	El Caballo (10)	70JB09_10	-	KC625839 KC625840	KC625712	KC625611	37,0134	-3,4405
L. glacialis Boiss.	Puntal de los Cuartos (1)	76JB09_1	-	-	KC625769	KC625668	37,1132	-3,2758
L. glacialis Boiss.	Puntal de los Cuartos (2)	76JB09_2	-	KC625841 KC625842	KC625713	KC625612	37,1135	-3,2757
L. glacialis Boiss.	Puntal de los Cuartos (3)	76JB09_3	-	KC625849 KC625850	KC625717	KC625616	37,1127	-3,2757
L. glacialis Boiss.	Puntal de los Cuartos (4)	76JB09_5	-	KC625905 KC625906	KC625747	KC625646	37,1123	-3,2752
L. glacialis Boiss.	Puntal de los Cuartos (5)	76JB09_6	-	- -	KC625753	KC625652	37,1123	-3,2752
L. glacialis Boiss.	Puntal de los Cuartos (6)	76JB09_7	-	KC625925 KC625926	KC625758	KC625657	37,1120	-3,2751
L. glacialis Boiss.	Puntal de los Cuartos (7)	76JB09_9	-	KC625937 KC625938	KC625764	KC625663	37,1114	-3,2745

L. glacialis Boiss.	Puntal de los Cuartos (8)	76JB09_10	-	KC625945 KC625946	KC625768	KC625667	37,1100	-3,2758
L. glacialis Boiss.	Puntal de los Cuartos (9)	76JB09_11	-	- -	KC625770	KC625669	37,1112	-3,2746
L. glacialis Boiss.	Puntal de los Cuartos (10)	76JB09_12	-	KC625817 KC625818	KC625701	KC625600	37,1180	-3,2800
L. glacialis Boiss.	Cerro de San Juan (1)	62JB09_ 1	-	KC625913 KC625914	KC625751	KC625650	37,0797	-3,3644
L. glacialis Boiss.	Cerro de San Juan (2)	62JB09_ 2	-	KC625915 KC625916	KC625752	KC625651	37,0803	-3,3645
L. glacialis Boiss.	Cerro de San Juan (3)	62JB09_ 3	-	KC625923 KC625924	KC625757	KC625656	37,0805	-3,3646
L. glacialis Boiss.	Cerro de San Juan (4)	62JB09_ 4	-	KC625909 KC625910	KC625749	KC625648	37,0802	-3,3648
L. glacialis Boiss.	Cerro de San Juan (5)	62JB09_ 5	-	KC625917 KC625918	KC625754	KC625653	37,0794	-3,3644
L. glacialis Boiss.	Cerro de San Juan (6)	62JB09_ 6	-	KC625927 KC625928	KC625759	KC625658	37,0801	-3,3645
L. glacialis Boiss.	Cerro de San Juan (7)	62JB09_ 7	-	KC625984 KC625985	KC625791	KC625690	37,0809	-3,3651
L. glacialis Boiss.	Pico del Cuervo (1)	173JB10_1	-	KC625961 KC625962	KC625778	KC625677	37,0942	-3,2820
L. glacialis Boiss.	Pico del Cuervo (2)	173JB10_2	-	KC625967 KC625968	KC625782	KC625681	37,0959	-3,2825
L. glacialis Boiss.	Pico del Cuervo (3)	173JB10_3	-	KC625975 KC625976	KC625785	KC625684	37,0959	-3,2825
L. glacialis Boiss.	Pico del Cuervo (4)	173JB10_4	-	KC625981 KC625982	KC625789	KC625688	37,0961	-3,2830
L. glacialis Boiss.	Pico del Cuervo (5)	173JB10_5	-	KC625988 KC625989	KC625793	KC625692	37,0961	-3,2830
L. glacialis Boiss.	Puntal de Vacares (6)	173JB10_6	-	KC625831 KC625832	KC625708	KC625607	37,0966	-3,2851
L. glacialis Boiss.	Puntal de Vacares (7)	173JB10_7	-	KC625837 KC625838	KC625711	KC625610	37,0969	-3,2870
L. glacialis Boiss.	Puntal de Vacares (8)	171JB10_1	-	KC625813 KC625814	KC625698	KC625597	37,0809	-3,2881
L. glacialis Boiss.	Puntal de Vacares (9)	171JB10_2	-	KC625823	KC625704	KC625603	37,0809	-3,2881

				KC625824				
L. glacialis Boiss.	Puntal de Vacares (10)	171JB10_3	-	KC625829 KC625830	KC625707	KC625606	37,0814	-3,2883
L. glacialis Boiss.	Puntal de Vacares (11)	171JB10_4	-	KC625835 KC625836	KC625710	KC625609	37,0857	-3,2856
L. glacialis Boiss.	Puntal de Vacares (12)	171JB10_5	-	KC625845 KC625846	KC625715	KC625614	37,0859	-3,2858
L. glacialis Boiss.	Pico del Globo (1)	169JB10_1	-	KC625853 KC625854	KC625719	KC625618	37,0594	-3,2953
L. glacialis Boiss.	Pico del Globo (2)	169JB10_2	-	KC625847 KC625848	KC625716	KC625615	37,0591	-3,2946
L. glacialis Boiss.	Pico del Globo (3)	169JB10_3	-	KC625861 KC625862	KC625723	KC625622	37,0591	-3,2946
L. glacialis Boiss.	Pico del Globo (4)	169JB10_5	-	KC625869 KC625870	KC625727	KC625626	37,0594	-3,2954
L. glacialis Boiss.	Pico del Globo (5)	169JB10_6	-	KC625855 KC625856	KC625720	KC625619	37,0594	-3,2954
L. glacialis Boiss.	Pico del Globo (6)	169JB10_7	-	KC625877 KC625878	KC625732	KC625631	37,0595	-3,2957
L. glacialis Boiss.	Pico del Globo (7)	169JB10_12	-	- -	-	-	37,0591	-3,2944
L. glacialis Boiss.	Pico del Globo (8)	169JB10_9	-	KC625885 KC625886	KC625736	KC625635	37,0591	-3,2944
L. glacialis Boiss.	Pico del Globo (9)	169JB10_10	-	KC625863 KC625864	KC625724	KC625623	37,0625	-3,2964
L. glacialis Boiss.	Pico del Globo (10)	169JB10_11	-	KC625871 KC625872	KC625728	KC625627	37,0629	-3,2962

Supplementary table 3. Allele specific primers (ASP) designed for the amplification of AGT1 haplotypes of dataset B.

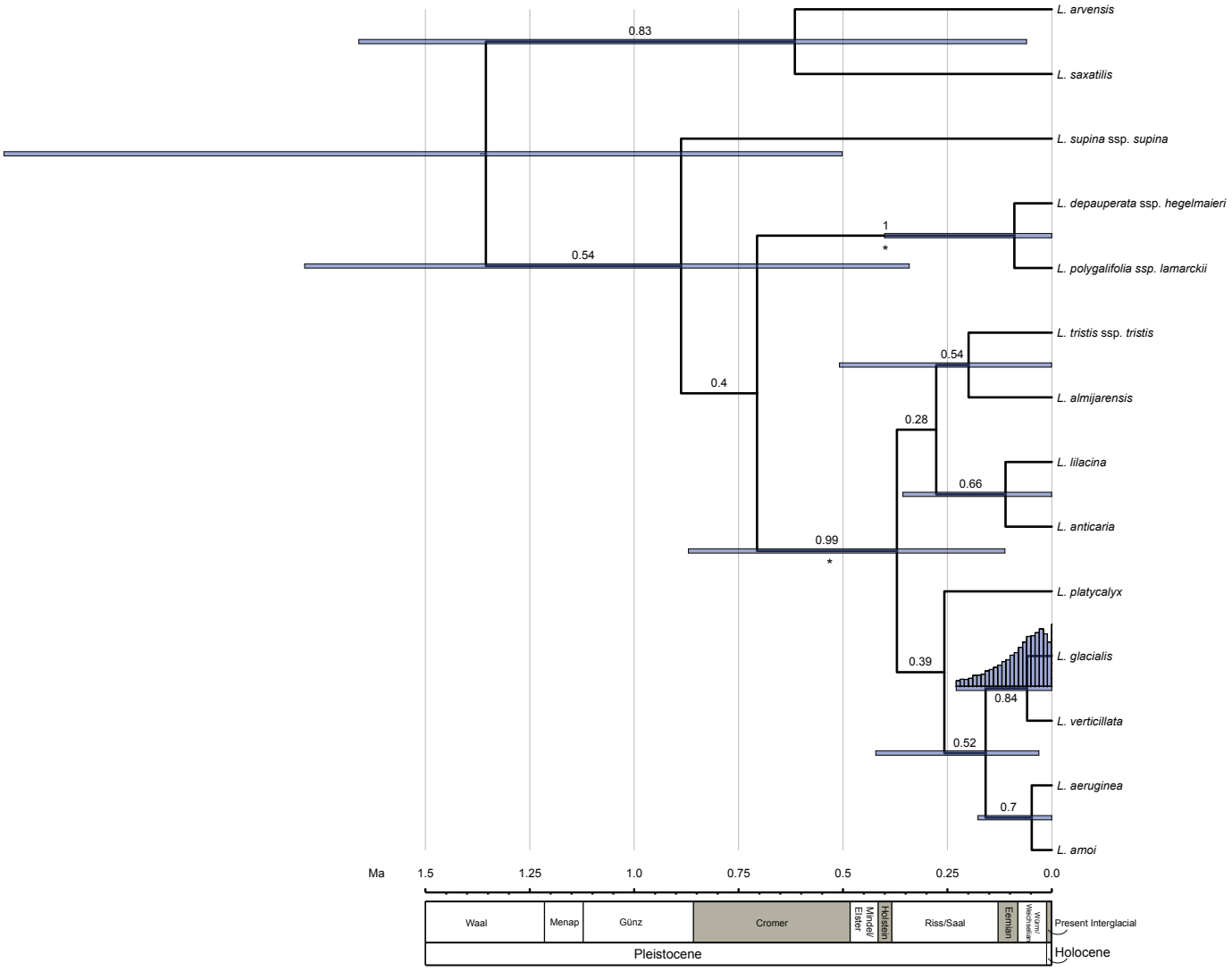
AGT1_ASP1_C	GGCTCTTGAGGCC
AGT1_ASP1_A	GGCTCTTGAGGCA
AGT1_ASP2_C	TGGTCTGGGAATC
AGT1_ASP2_T	TGGTCTGGGAATT
AGT1_ASP3_C	AGGACTCGACAAC
AGT1_ASP3_T	AGGACTCGACAAT
AGT1_ASP4_C	GAGAGTTTTCTTC
AGT1_ASP4_T	GAGAGTTTTCTTT
AGT1_ASP5_G	CTATTTGGACATG
AGT1_ASP5_T	CTATTTGGACATT
AGT1_ASP6_G	GGGCGATTACTTG
AGT1_ASP6_A	GGGCGATTACTTA

References

Liepman AH, Olsen LJ (2001) Peroxisomal alanine : glyoxylate aminotransferase (AGT1) is a photorespiratory enzyme with multiple substrates in *Arabidopsis thaliana*. *The Plant Journal* **25**, 487-498.

Shaw J, *et al.* (2007) Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: The Tortoise and the hare III. *American Journal of Botany* **94**, 275-288.

White TJ, Bruns, T., Lee, S. and Taylor, J. (1990) *Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In "PCR Protocols. A Guide to Methods and Applications"* Academic Press, San Diego. USA.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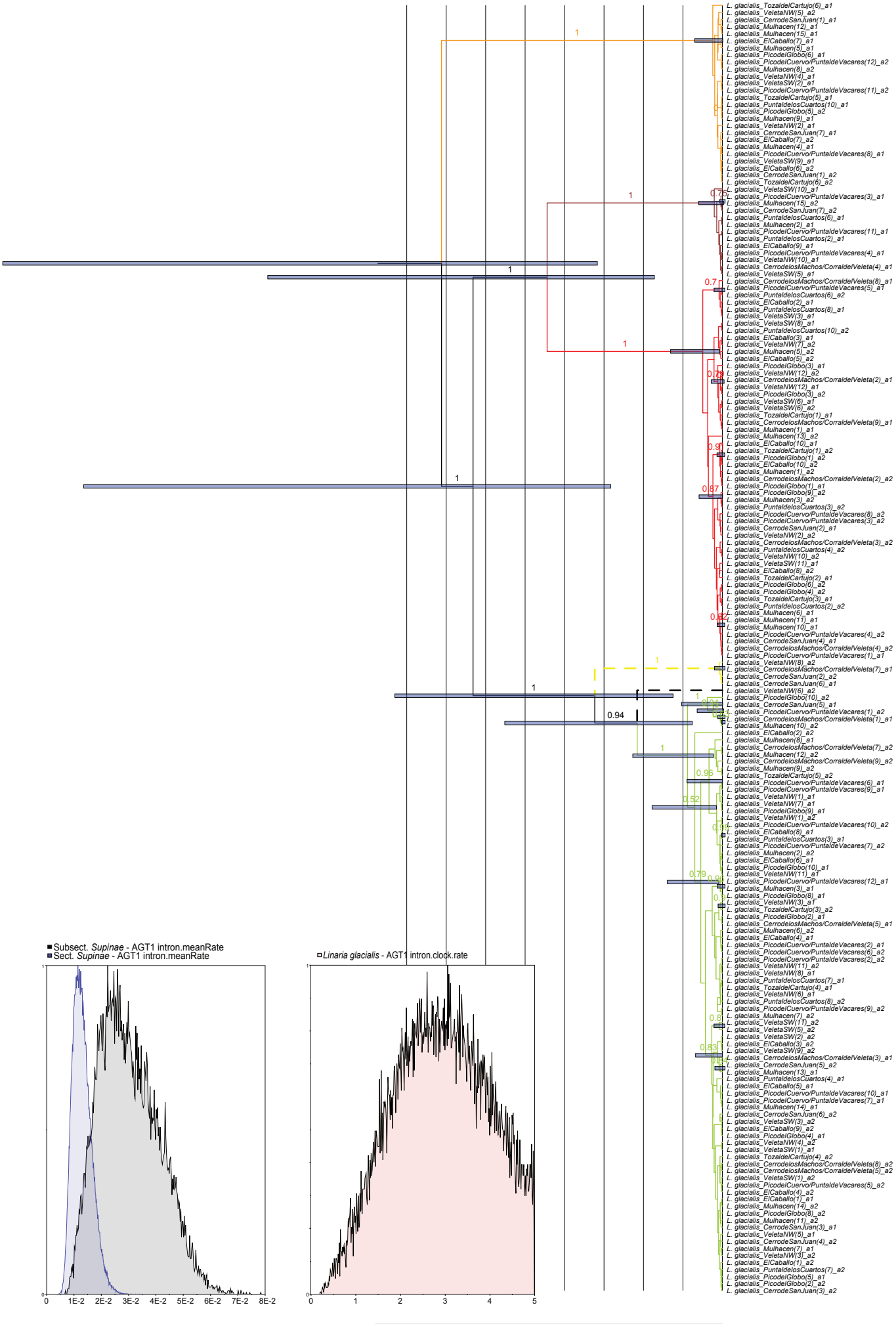
L. tristis ssp. tristis
0.54
0.4
L. almijarensis
0.28
L. lilacina
0.66
L. anticaria
0.99
*
L. platycalyx
L. glacialis
0.39
0.84
L. verticillata
0.52
L. aeruginea
0.7
L. amoi
Ma
1.5
1.25
1.0
0.75
0.5
0.25
0.0
Waal
Menap
Günz
Cromer
Mindel/ Elster
Holstein
Riss/Saal
Eemian
Würm/ Weichselian
Present Interglacial
Pleistocene
Holocene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0.7
0.96
0.87
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0.83
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Sect. Supinae - AGT1 intron.meanRate
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8E-2
Linaria glacialis - AGT1 intron.clock.rate
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2
3
4
5
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15000
10000
5000
0
years ago

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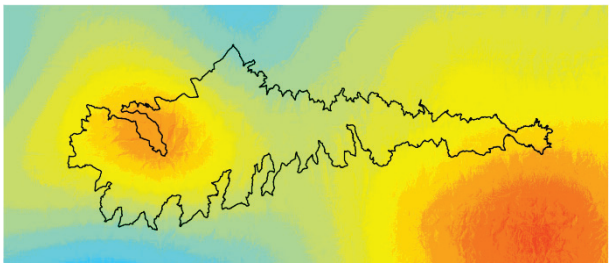

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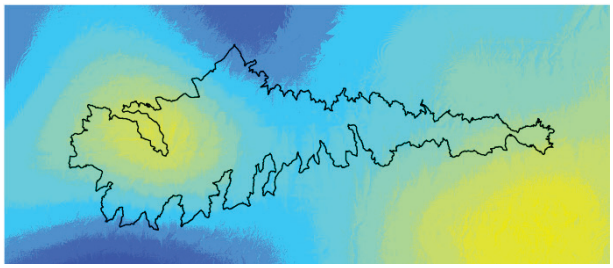

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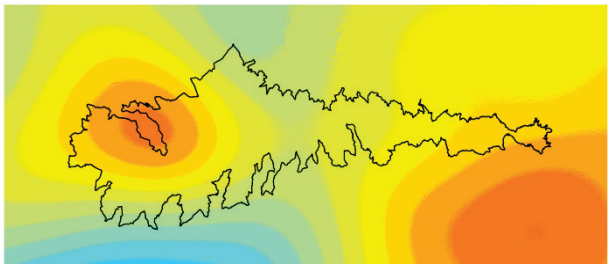

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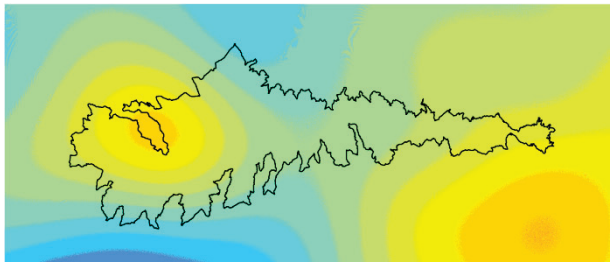
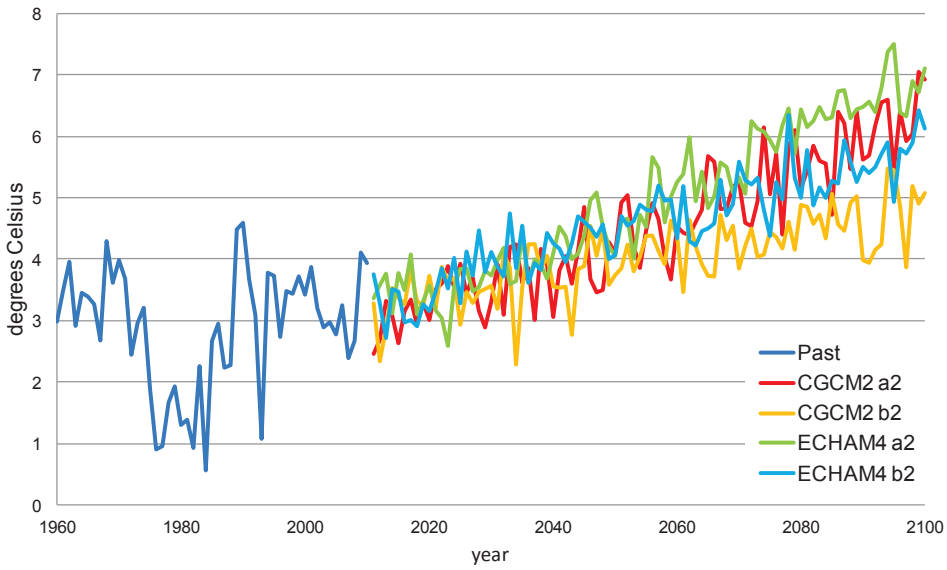


E. ALL SCENARIOS



Supplementary materials and methods

Study species

Linaria glacialis Boiss. (Fam. Plantaginaceae, tribe Antirrhineae) is an annual or perennial narrow endemic toadflax inhabiting schistose screes of the alpine vegetation belt of Sierra Nevada (2700-3400 m). It has an occluded corolla similar to that of snapdragons (*Antirrhinum*), but with a long spur. The corolla is similar in size and shape to that of closely related congeneric species (subsect. Supinae, sect. Supinae; Blanco-Pastor *et al.* 2012). *L. glacialis* is mainly differentiated from other *Linaria* species by its large leaf-like calyx lobes and bracts (see Fig. 1B). The conservation status of this species is Vulnerable –VU D2; UICN category– (Blanca *et al.* 1998) and the number of individuals has been estimated to be less than 10,000 (Blanca *et al.* 2001). The scarcity of adequate habitat is considered the main threat for this species (Blanca *et al.* 1998; Blanca *et al.* 2001).

Study area

Sierra Nevada is located in the southern part of Spain nearby the Mediterranean Sea (see Fig. 1A) and has the highest altitude of the Iberian Peninsula (Mulhacen, 3481 m a.s.l.). This mountain range was covered with glaciers only in areas above ~2,500 m a.s.l. during Quaternary glaciations (Gómez-Ortiz *et al.* 1996), while large areas remained free of permanent ice. Sierra Nevada was a refuge area for many European species during glacial ages (Blanca *et al.* 1998; González-Sampériz *et al.* 2010). Indeed, location and altitude of Sierra Nevada has interested scientist to test speciation patterns in plants (Gutiérrez Larena *et al.* 2002; Kropf *et al.* 2006).

The upper vegetation belt (alpine or cryoromediterranean) of Sierra Nevada has an extension of 3875,7 ha and its lower boundaries span between 2750 m in northern and western zones and 3290 m in southern and eastern zones (Fernández Calzado & Molero Mesa 2011). It shelters an abrupt landscape with steep slopes, screes and rocky areas and has a metamorphic substrate composed by feldspar, micaschist, slates and quartzites. The effect of glacier phenomena is still present in the northern part of the range, although at the beginning of 20th century the last ice mass (Veleta glacier) was strongly reduced and since 1995 there are no traces left (Gómez-Ortiz *et al.* 2009). In this area the vegetation is exposed to stressful climate conditions with large daily temperature oscillations and a pronounced summer drought.

Sampling and DNA sequencing strategy

Apart from the *L. glacialis* dataset, an additional dataset (species-level dataset) was generated for the present study. The species-level dataset included DNA sequences of two *L. glacialis* individuals and 15 individuals of other closely-related species, which were chosen based on previous phylogenetic studies (Blanco-Pastor *et al.* 2012; Fernández-Mazuecos *et al.* 2013): *L. aeruginea (*two individuals), *L. almijarensis*, *L. anticaria*, *L. lilacina*, *L. platycalyx*, *L. tristis*, *L. amoi*, *L. verticillata*, *L. depauperata*, *L. polygalifolia (*3 individuals), *L. supina*, *L. arvensis (*two individuals) and *L. saxatilis* (see above). The species-level dataset comprised both previously published and newly generated sequences of the ITS region (Blanco-Pastor *et al.* 2012; Fernández-Mazuecos *et al.* 2013) and the low copy nuclear gene AGT1 (partial intron) (Blanco-Pastor *et al.* 2012) (see Supplementary table 1 and 2).

Deciphering of haplotypes from unphased genotypes and test for recombination

In addition to AGT1 sequences of the *L. glacialis* dataset, AGT1 and ITS sequences of the species-level dataset were also analyzed with PHASE. In the species-level dataset, when haplotypes inferred were not statistically supported, we obtained haplotype data by cloning the PCR products as done in Blanco-Pastor *et al.* (2012). In the cloning process several paralogous copies were obtained for the ITS region from one species (*L. aeruginea*) and for the AGT1 region from another species (*L. verticillata*). We then assessed the orthology of amplification products following Whittall *et al.* (2006).

Recombination was also tested within the AGT1 and ITS alignments of the species-level dataset using the software RDP 3.44 (Martin *et al.* 2010). Methods and parameter settings were as described in Blanco-Pastor *et al.* (2012).

Species tree estimation

In order to investigate the evolutionary framework for *L. glacialis* we performed a *BEAST species tree analysis (Heled & Drummond 2010) as implemented in the BEAST software v.1.7.2 (Drummond & Rambaut 2007; Drummond *et al.* 2012) using ITS and AGT1 intron haplotype sequences of the species-level dataset after excluding two AGT1 haplotypes with hard incongruence (*L. amoi*, *L. lilacina*) subject to be caused by hybridization/introgression (as the *BEAST model does not account for such processes). The crown age of section *Supinae* was calibrated with a normal distribution prior with mean 1.95 Ma and standard deviation 0.66. This date was previously obtained in a three-locus species tree phylogeny of *Linaria* sect. *Supinae* with detection and exclusion of plausible hybrids (Blanco-Pastor *et al.* 2012). The remaining prior settings of the analysis were equal to those used in that study.

Population size history

Extended Bayesian skyline plot

Extended Bayesian skyline plot (EBSP) analysis allows the joint analysis of multiple loci and uses Bayesian stochastic variable selection to select the appropriate smoothness of the demographic function (number of groups of coalescent intervals). The analysis was carried out combining information of the four regions of the *L. glacialis* dataset two linked plastid loci (ptDNA) and two linked regions of the AGT1 gene. We included the four regions as independent partitions for the analysis with distinct substitution models as obtained with jModeltest 0.1.1 (Posada 2008) (see Supplementary table 1). The two plastid regions were linked for the partition tree and the clock model priors. AGT1 exon and intron regions were also linked for the partition tree but unlinked for the clock model priors. Upper limits for the mean.rate parameters were set to 5 s/s/Ma for the AGT1 regions and 1 s/s/Ma for the ptDNA loci. Operators of the analysis were also modified following the recommendations of the EBSP tutorial available in http://beast.bio.ed.ac.uk/Tutorials.

Supplementary results

Haplotype data gathering and recombination test

The species-level dataset included 36 ITS and 33 AGT1 sequences. Recombination was neither detected in the ITS or AGT1 alignments of the species-level dataset by any of the five methods used. This allowed us to perform the species tree analysis without discarding any recombinant region.

Species tree

Despite the low resolution and the requirement of additional loci to unravel the species relationships within this group (sect. *Supinae* subsect. *Supinae*), the higher number of species and sequences used here compared to the analysis of Blanco-Pastor *et al.* (2012) extends the previous phylogenetic information obtained of this recently diversified group of *Linaria*. The species tree obtained from dataset A (Supplementary Fig. 1) showed *L. glacialis* within subsect. *Supinae sensu* Blanco-Pastor *et al.* (2012) and forming a monophyletic group together with eight southern Iberian endemics (0.99 PP). Additionally it also showed *L. glacialis* as sister to the sub-endemic low-land species of Sierra Nevada *L. verticillata* (with moderate support; 0.84 PP) (see Supplementary Fig. 1), with a divergence time 0 – 230 ka 95% HPD (60 ka median).

References

Blanca G, *et al.* (1998) Threatened vascular flora of Sierra Nevada (southern Spain). *Biological Conservation* **85**, 269-285.

Blanca G, *et al.* (2001) *Flora Amenazada y Endémica de Sierra Nevada* Consejería de Medio Ambiente. Junta de Andalucía, Granada.

Blanco-Pastor JL, *et al.* (2012) Coalescent Simulations Reveal Hybridization and Incomplete Lineage Sorting in Mediterranean *Linaria*. *Plos One* **7**, e39089.

Drummond A, Rambaut A (2007) BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology* **7**, 214.

Drummond AJ, *et al.* (2012) Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology and Evolution* **29**, 1969-1973.

Fernández-Mazuecos M, *et al.* (2013) A Phylogeny of Toadflaxes (Linaria Mill.) Based on Nuclear Internal Transcribed Spacer Sequences: Systematic and Evolutionary Consequences. *International Journal of Plant Sciences* **174**, 234-249.

Fernández Calzado MR, Molero Mesa J (2011) The cartography of vegetation in the cryoromediterranean belt of Sierra Nevada: a tool for biodiversity conservation. *LAZAROA* **32**, 101-115.

Gómez-Ortiz A, *et al.* (2009) Evidences from historical documents of landscape evolution after Little Ice Age of a Mediterranean high mountain area, Sierra Nevada, Spain (eighteenth to twentieth centuries). *Geografiska Annaler: Series A, Physical Geography* **91**, 279-289.

Gómez-Ortiz A, *et al.* (1996) Contribución al conocimiento de la deglaciación reciente y morfología asociada del Corral del Veleta (Sierra Nevada). *Cadernos do Laboratorio Xeolóxico de Laxe* **21**, 543-558.

González-Sampériz P, *et al.* (2010) Steppes, savannahs, forests and phytodiversity reservoirs during the Pleistocene in the Iberian Peninsula. *Review of Palaeobotany and Palynology* **162**, 427-457.

Gutiérrez Larena B, *et al.* (2002) Glacial-induced altitudinal migrations in *Armeria* (Plumbaginaceae) inferred from patterns of chloroplast DNA haplotype sharing. *Molecular Ecology* **11**, 1965-1974.

Heled J, Drummond AJ (2010) Bayesian Inference of Species Trees from Multilocus Data. *Molecular Biology and Evolution* **27**, 570-580.

Kropf M, *et al.* (2006) Long-distance dispersal vs vicariance: the origin and genetic diversity of alpine plants in the Spanish Sierra Nevada. *New Phytologist* **172**, 169-184.

Martin DP, *et al.* (2010) RDP3: a flexible and fast computer program for analyzing recombination. *Bioinformatics* **26**, 2462-2463.

Posada D (2008) jModelTest: Phylogenetic model averaging. *Molecular Biology and Evolution* **25**, 1253-1256.

Whittall JB, *et al.* (2006) Generating single-copy nuclear gene data for a recent adaptive radiation. *Molecular Phylogenetics and Evolution* **39**, 124-134.

DISCUSIÓN

SISTEMÁTICA DE *LINARIA* SECT. *SUPINAE*

MONOFILIA DE LA SECCIÓN Y RELACIÓN CON OTRAS SECCIONES

En relación a la taxonomía de *Linaria,* varios expertos (Morison 1680; Lange 1870; Boissier 1879; Wettstein 1895; Viano 1978a, 1978b) consideraron que el género debía ser dividido en dos grupos naturales diferenciados por el tipo de semilla (alada o áptera). El análisis de *Linaria* (basado en secuencias ITS) mostrado en el Manuscrito 1 (Fernández-Mazuecos *et al.* 2013b), así como los análisis centrados en la sect. *Supinae* con un grupo externo representativo de todo el género (dos regiones nucleares y 2-3 regiones plastidiales) (Manuscritos 2 y 3, Blanco-Pastor *et al.* 2012; Blanco-Pastor & Vargas 2013), revelan que el cambio hacia un mismo tipo de semilla ha ocurrido varias veces de forma independiente durante la historia evolutiva del género. Los análisis filogenéticos bajo el modelo de coalescencia de multiespecies (*Multispecies coalescent model*) (Blanco-Pastor *et al.* 2012; Blanco-Pastor & Vargas 2013) indican que las especies de la sect. *Supinae* del género *Linaria* formarían un grupo monofilético. Pese a la incertidumbre en las relaciones entre las distintas secciones, la sect. *Supinae* no estaría directamente emparentada con las secciones *Linaria* y *Pelisserianae* que también presentan semillas aladas. Ello fue sugerido por autores previos (Valdés 1970a; Sutton 1988) debido a la anatomía no homóloga de las semillas aladas de la sect. *Pelisserianae* con respecto a las secciones *Supinae* y *Linaria*. Pese a las similitudes en la forma de la semilla entre las secciones *Supinae* (mayoritariamente semillas aladas) y *Linaria* (semillas aladas), la filogenia ITS muestra que la sect. *Linaria* queda más emparentada a la sect. *Speciosae* de semillas ápteras, mientras que la sect. *Supinae* está más emparentada a la sect. *Diffusae* (*L. reflexa* group), también de semillas ápteras (Manuscrito 1, Fernández-Mazuecos *et al.* 2013b). Sin embargo otros caracteres morfológicos sí que apoyan las relaciones obtenidas a partir de las secuencias ITS: (i) tallos, hojas, cápsula y flores similares en las secciones *Linaria* y *Speciosae* y (ii) presencia de tallos primarios fértiles y secundarios estériles en sect. *Supinae* y sect. *Diffusae*. Análisis específicos como los realizados en el Manuscrito 2 (Blanco-Pastor *et al.* 2012) serán necesarios para someter a test posibles eventos de hibridación entre secciones que hayan podido determinar la topología observada al analizar las secuencias ITS (Manuscrito 1, Fernández-Mazuecos *et al.* 2013b).

CLASIFICACIÓN INTERNA DE LA SECCIÓN *SUPINAE*

Pese a la monofilia obtenida para la sect. *Supinae* en el Manuscrito 2 (Blanco-Pastor *et al.* 2012), hay que tener en cuenta la incertidumbre en el origen de ciertas especies como *L. latifolia* (sect. *Supinae*) y

L. hirta (sect. *Diffusae*). Ambas especies presentan posiciones basales al resto de especies de la sect. *Supinae* en el árbol ITS, siendo la primera de posición más basal (Manuscrito 1, Fernández-Mazuecos *et al.* 2013b). Dado que *L. latifolia* presenta caracteres similares a *L. hirta* es posible que la especie tenga un origen híbrido fruto del cruce entre algún progenitor de la sect. *Supinae* y otro de la sect. *Diffusae*. El origen híbrido de esta especie no se ha podido someter a test específicamente en este trabajo (debido a la dificultad en la secuenciación de la región AGT1 en ambas especies). Análisis específicos serán de gran interés para demostrar la capacidad de generación de especies híbridas mediante el cruce entre especies de la sect. *Supinae* y especies de otras secciones, tal y como se ha mostrado en tratamientos experimentales (Valdés 1970b).

Según los análisis del Manuscrito 2 (Blanco-Pastor *et al.* 2012) la sect. *Supinae* quedaría dividida en tres grupos (subsect. *Supinae*, subsect. *Saxatile* y subsect. *Arvenses*). Estas tres subsecciones se diferencian morfológicamente mediante tres caracteres: (i) longevidad, (ii) tamaño de la corola y (iii) tipo de ala de las semillas. Ciertas especies (*L. orbensis*, *L. saturejoides* y *L. oblongifolia*) tienen características típicas de dos de las subsecciones, lo cual apoya su origen híbrido tal y como ha sido reflejado en la reconstrucción del árbol de especies multietiqueta (*multilabelled species tree*).

La hibridación juega un papel fundamental en la generación de especies de plantas (Rieseberg 1997; Seehausen 2004; Soltis & Soltis 2009). Sin embargo, la evolución reticulada afecta de forma muy importante tanto a la clasificación sistemática a niveles terminales mediante el uso de filogenias moleculares (Rieseberg & Soltis 1991) como a las delimitaciones taxonómicas. De esta forma se ha encontrado incongruencia filogenética y taxonómica en numerosos grupos de plantas de divergencia reciente, en los que la hibridación ha debido jugar un papel muy importante (Comes & Abbott 2001; Guo *et al.* 2004; Albaladejo *et al.* 2005; Mansion *et al.* 2005; Valcárcel *et al.* 2006; Maureira-Butler *et al.* 2008; Frajman *et al.* 2009; Presti *et al.* 2010; Barres *et al.* 2011; Wilson & Hudson 2011). En muchos grupos de plantas, la complejidad y falta de consenso en la delimitación taxonomica parece ser la regla en lugar de la excepción. Para la mayoría de estos grupos, la evolución reticulada es un factor fundamental a tener en cuenta para afrontar las clasificaciones sistemáticas. La presente memoria de tesis doctoral manifiesta la importancia del estudio de la hibridación como proceso generador de patrones complejos de diversidad y de relaciones evolutivas. La comprensión y reconstrucción de eventos de hibridación deberá ser de carácter prioritario en estos grupos de especies. Esto es tan relevante como la clasificación y delimitación taxonómica en entidades discretas (subespecies, especies, subsecciones). Debido a que la delimitación taxonómica en grupos que han podido sufrir hibridación podría ser especialmente problemática a la hora de establecer programas de investigación y conservación (Ennos *et al.* 2005).

EVOLUCIÓN DE *LINARIA* SECT. *SUPINAE* DURANTE EL CUATERNARIO

SÍNTESIS DE LA HISTORIA EVOLUTIVA

Los Manuscritos 2-5 (Blanco-Pastor *et al.* 2012; Blanco-Pastor *et al.* 2013; Blanco-Pastor & Vargas 2013) nos permiten realizar una reconstrucción sintética de la evolución de *Linaria* sect. *Supinae* en un marco temporal. Esto ha sido posible gracias a los análisis de datación basados en el uso del reloj molecular relajado (Drummond *et al.* 2006), junto con calibraciones secundarias [basadas en un análisis calibrado con fósiles de familias y tribus del orden Lamiales (Appendix 1, Vargas *et al. in press*)]. Se ha comprobado que el ancestro común de la sect. *Supinae* data del Plioceno tardío – Pleistoceno reciente (0.87 – 3.28 Ma) (Manuscrito 2, Blanco-Pastor *et al.* 2012), período en el que se inició la sucesión de ciclos glaciales típicos del Cuaternario (Webb & Bartlein 1992) y en el que ya se había establecido el clima mediterráneo, con veranos muy secos (Suc 1984). En la Península Ibérica, centro de diversificación de *Linaria* sect. *Supinae*, las principales presiones abióticas para las plantas han debido ser tanto las condiciones xéricas típicas de la estacionalidad anual, como las oscilaciones climáticas de las glaciaciones.

La reconstrucción de la evolución en *Linaria* sect. *Supinae* nos ha permitido confirmar la hipótesis propuesta en la introducción de la presente memoria de tesis doctoral: *Los patrones evolutivos de Linaria sect. Supinae a distintas escalas temporales y taxonómicas han estado determinados en gran medida por las estrategias reproductivas de las especies y el marco geográfico de aislamiento*. La diferenciación de los dos linajes más diversos de la sect. *Supinae* (subsect. *Supinae* y subsect. *Saxatile*) muestra un patrón geográfico en la Península Ibérica, tal y como indica la distribución de los haplotipos plastidiales del Manuscrito 3 (Blanco-Pastor & Vargas 2013). También, el aislamiento reproductivo por autogamia (e.g. Martin & Willis 2007; Guo *et al.* 2009; Grossenbacher & Whittall 2011) ha podido ser importante en la diferenciación del linaje formado por las especies de la subsect. *Arvenses*, ya que los principales caracteres sinapomórficos de esta sección son la autocompatibilidad y el pequeño tamaño de las flores (Manuscrito 3, Blanco-Pastor & Vargas 2013).

En los linajes más terminales, el cambio en la distribución de las especies siguiendo el modelo de contracción-expansión (Hewitt 1996) (típico de las oscilaciones climáticas del Cuaternario) ha debido configurar la evolución en *Linaria* sect. *Supinae*. Procesos de aislamiento geográfico promovidos por los ciclos glaciales y la heterogeneidad orográfica de la Península Ibérica han debido intercalarse con eventos de mezcla de las poblaciones dando lugar a formación de especies híbridas o introgresión. Estos cruzamientos parecen haber sido más importantes en el linaje de la subsect. *Saxatile* (Manuscrito 2, Blanco-Pastor *et al.* 2012), que presenta una mayor diversidad en la

mitad norte de la Península Ibérica. Esta región parece haber sido más afectada por las oscilaciones climáticas que las zonas del sur de la Península Ibérica (Hewitt 2001; Feliner 2011; Manuscrito 5, Blanco-Pastor *et al.* 2013). Sin embargo, durante los cambios en la distribución de las especies, también se han debido formar especies híbridas mediante cruces entre la subsect. *Saxatile* y la subsect. *Supinae*, tal y como muestra la reconstrucción del origen de ciertos linajes híbridos (*L. orbensis*, *L. oblongifolia* y *L. saturejoides*) (Manuscrito 2, Blanco-Pastor *et al.* 2012).

En la sect. *Supinae*, otros promotores de la diferenciación rápida en ciertos linajes alógamos durante las últimas etapas del Cuaternario han debido ser el aislamiento geográfico, tal y como muestra la distribución de haplotipos plastidiales (Manuscrito 2, Blanco-Pastor *et al.* 2012), junto con el refuerzo de la diferenciación promovido por los polinizadores locales. De esta forma encontramos un mínimo de 18 especies alógamas endémicas o subendémicas de la Península Ibérica (*L. tristis, L. atrofusca, L. caesia, L. supina, L. depauperata, L. polygalifolia, L. aeruginea, L. platycalyx, L. almijarensis, L. cuartanensis, L. amoi, L. anticaria, L. verticillata, L. lilacina, L. oligantha, L. filicaulis, L. amethystea, L. saxatilis*), que difieren en la fauna polinizadora a pesar de su proximidad geográfica. En este sentido, análisis de la forma floral en diez de estas especies originadas en una radiación (subsect. *Supinae*) han mostrado una especialización a ciertas especies de polinizadores (Manuscrito 5, Blanco-Pastor *et al. submitted*).

Por otro lado, encontramos tres especies autógamas estrictas (*L. arvensis*, *L. simplex*, *L. micrantha,* subsect. *Arvenses*) que han mantenido una limitada capacidad de diferenciación. Sin embargo, gracias a una combinación de caracteres tales como: (i) autocompatibilidad, (ii) corto período de vida y (iii) desarrollo en sustratos no específicos, las tres especies han podido expandir su área de distribución a lo largo de toda la cuenca mediterránea y Europa (Manuscrito 3, Blanco-Pastor & Vargas 2013). A pesar de que la disminución en el nivel del mar durante períodos glaciales debió facilitar las conexiones entre el sur de Europa y norte de África (Rodríguez-Sánchez *et al.* 2008; Lo Presti & Oberprieler 2011), las especies con amplia distribución de la subsect. *Arvenses* han tenido que superar masas de agua y otras barreras geográficas presentes a lo largo de la cuenca del Mediterráneo. De esta forma, estas especies que comparten características autoecológicas propicias para la colonización de areas remotas, han podido abarcar en un corto período de tiempo (< 0.72 Ma) un área cuyos extremos se encuentran a más de 8000 km de distancia.

Una especie autocompatible de las cumbres alpinas de Sierra Nevada (*Linaria glacialis*) (>3000 m), con un origen y diferenciación muy reciente en el Cuaternario (0 – 0.23 Ma), ha podido resistir las oscilaciones climáticas de este período. La distribución de esta especie ha sufrido pequeñas

oscilaciones durante los últimos miles de años y su tamaño efectivo poblacional ha sido levemente afectado durante el último milenio (Manuscrito 5, Blanco-Pastor *et al.* 2013). Las poblaciones de esta especie han demostrado tener una exitosa adaptación a las condiciones locales. De esta forma, un sistema reproductivo mixto (autocompatible pero con gran tamaño floral e índice P/O típico de especies autoincompatibles) ha debido jugar un papel fundamental en la seguridad reproductiva, así como en la variabilidad genética, que está homogéneamente repartida a lo largo de su área de distribución (Manuscrito 5, Blanco-Pastor *et al.* 2013).

FACTORES RESPONSABLES DE LA COLONIZACIÓN Y SUPERVIVENCIA

En la sect. *Supinae*, observamos ocho especies que se han clasificado como predominantemente autógamas (*L. glacialis*, *L. alpina*, *L. arenaria*, *L. tursica*, *L. arvensis*, *L. simplex*, *L. micrantha*, véase Manuscrito 3; Blanco-Pastor & Vargas 2013). El análisis filogeográfico del ADN plastidial de *L. arvensis*, *L. simplex* y *L. micrantha* (tres especies de amplia distribución pertenecientes a la subsect. *Arvenses*) mostró un flujo genético dinámico ya que la diferenciación estaba poco relacionada por la geografía y se detectaron eventos de dispersión a larga distancia. El análisis en detalle de la diversidad genética en poblaciones de *L. glacialis* (especie autocompatible de la subsect. *Supinae* endémica de las cumbres de Sierra Nevada) también mostró una estructura geográfica insignificante, con una variación principalmente explicada por la variabilidad intrapoblacional.

Las transiciones de autoincompatibilidad a autocompatibilidad han sido muy frecuentes en las angiospermas, algo que ha sido demostrado a partir de análisis genéticos, ecológicos y filogenéticos (Stebbins 1974; Grant 1981; Igic *et al.* 2008; Busch *et al.* 2011). Sin embargo, se ha argumentado que la evolución hacia un sistema de autogamia constituye un paso atrás en términos evolutivos por la pérdida de potencial adaptativo asociado a variación genética (Darlington 1958; Lowry & Lester 2006).

Los resultados de las últimas décadas han apoyado que la evolución de los sistemas reproductivos puede ser consecuencia de factores ecológicos, lo cual refleja las posibles ventajas adaptativas de la autogamia. Hoy sabemos que la evolución hacia la autocompatibilidad se dará siempre y cuando ésta constituya una ventaja selectiva. En este sentido, Baker (1955) y Stebbins (1957) ya indicaron que la transición hacia un sistema de autogamia debía ser de gran importancia para la formación de especies capaces de sobrevivir en condiciones poco favorables para la polinización como las dispersiones fuera de su área de distribución. Sin embargo, aún hoy sigue latente el antiguo debate establecido en *The genetics of colonizing species* (Mayr 1965) que enfrenta la seguridad reproductiva

de especies autógamas generalistas (Jain 1976; Rambuda & Johnson 2004; Randle *et al.* 2009), frente a la capacidad adaptativa por variabilidad genética (reducida en especies autógamas) (Carlquist 1966; Brennan *et al.* 2005; Lowry & Lester 2006), como factores promotores de la colonización. La autocompatibilidad parece conferir una ventaja adaptativa en condiciones adversas para la polinización cruzada, como ocurre tras la colonización de lugares remotos por pocos propágulos, o la reducción de la actividad polinizadora. Sin embargo, los análisis del Manuscrito 3 (Blanco-Pastor & Vargas 2013) indican que otras características adicionales (e.g. ciclo de vida corto y tolerancia a climas y substratos) se asocian a una capacidad colonizadora excepcional en la sect. *Supinae*. La combinación de estas características en las especies de la subsect. *Arvenses* (*L. arvensis*, *L. simplex* y *L. micrantha*) identifica a un fenotipo altamente exitoso en la colonización y supervivencia a partir de un número reducido de propágulos (Baker 1965).

El estudio microevolutivo en *L. glacialis* (subsect. *Supinae*) ha mostrado que el cambio hacia una elevada capacidad de autofecundación también ha debido ser fundamental para la supervivencia de sus poblaciones, ya que esta especie se distribuye en zonas con condiciones climáticas muy adversas (cumbres alpinas de Sierra Nevada) que se traducen en una importante limitación de polinizadores. En *L. glacialis*, un sistema de reproducción mixto ha debido favorecer tanto la persistencia por una producción asegurada de semillas como el aumento de diversidad genética por alogamia, obteniendo así un equilibrio entre supervivencia a corto y largo plazo, siendo ambas fundamentales para la viabilidad poblacional en ambientes extremos y cambiantes como ocurre en la alta montaña mediterránea.

FACTORES RESPONSABLES DE LA DIFERENCIACIÓN Y DIVERSIFICACIÓN

La variación en los sistemas reproductivos ha debido ser un factor fundamental para la selección individual dentro de las poblaciones (véase apartado anterior). Sin embargo, los sistemas reproductivos también deben asociarse con las diferencias en la selección de especies [(mecanismo macroevolutivo según Grantham (2007) y (Jablonski 2007)] (Holsinger 2000). Algunos autores afirman que la selección positiva de especies (mayor diversificación) favorecería a los linajes autoincompatibles (Goldberg *et al.* 2010). En este sentido, estudios que aplican modelos de diversificación asociada a ciertas características han sugerido que la alogamia podría aumentar la diversificación debido a una tasa de extinción inferior comparada con la de los linajes autógamos (Goldberg *et al.* 2010; Goldberg & Igić 2012). La mayor tasa de extinción de especies autógamas se explicaría por la menor habilidad para responder a cambios ambientales debido a su menor

heterocigosidad, recombinación y tamaño efectivo poblacional (Holsinger 2000). La autogamia aumenta la probabilidad de que alelos desfavorables o deletéreos se fijen en las poblaciones, lo que reduce la capacidad reproductiva de la población y reduce el tamaño poblacional. Este proceso, que puede dar lugar a la extinción de las poblaciones autógamas de forma irreversible, se denomina colapso mutacional (*mutational meltdown*) (Gabriel *et al.* 1993).

Por el contrario, datos morfológicos (Baker 1953, 1959b) y de genética poblacional (Hamrick & Godt 1996; Charlesworth & Pannell 2001; Fishman & Stratton 2004) han sugerido que la transición hacia la autocompatibilidad podría promover la diferenciación y la especiación. La autogamia puede facilitar la fijación de especies híbridas. Si además otros mecanismos extremos de autogamia, como la apomixis, actúan tras procesos de hibridación, se puede producir especiación en poco tiempo (e.g. *Limonium*, Lledó *et al.* 2005). También, se ha demostrado empíricamente que la autogamia podría promover la cladogénesis (Martin & Willis 2007; Guo *et al.* 2009; Grossenbacher & Whittall 2011). Sin embargo, se ha argumentado que la transición hacia la autogamia estricta por sí sola no puede ser considerada como promotora de la especiación por producir aislamiento individual y no poblacional, por lo que cada individuo autógamo conformaría un linaje independiente y aislado del resto de individuos (Coyne & Orr 2004). De esta forma, las barreras reproductivas generadas por autogamia estricta podrían tener poca importancia en la generación de especies si se encuentran bajo presiones selectivas similares ya que la selección estabilizadora actuará sobre estos linajes a lo largo del tiempo (Thoday & Gibson 1962; Endler 1973; Andrew *et al.* 2012). En este sentido, a pesar de la extensa área de distribución y el aislamiento reproductivo de las especies autógamas estrictas de la sección *Supinae* (*L. arvensis*, *L. simplex*, *L. micrantha*; subsect. *Arvenses*), éstas no se han diferenciado y han mantenido un fenotipo altamente adaptado a las condiciones mediterráneas (Manuscrito 3, Blanco-Pastor & Vargas 2013).

La mayor diversidad genética de las especies alógamas de la sect. *Supinae* (Segarra-Moragues & Mateu-Andres 2007) junto con la diversidad de presiones bióticas a las que se enfrentan estas especies (Sánchez-Lafuente 2007; Sánchez-Lafuente *et al.* 2011) sugieren un papel activo de la alogamia en la especiación (Manuscrito 3, Blanco-Pastor & Vargas 2013) que no coincide con lo propuesto para otras angiospermas (Goldberg *et al.* 2010; Goldberg & Igić 2012). En definitiva, la sect. *Supinae* ha resultado ser un grupo de estudio muy interesante ya que encontramos diferentes patrones de diferenciación y diversificación asociados al sistema reproductivo.

Otros factores que han actuado en la diferenciación de la sect. *Supinae* resultan fundamentales para comprender la diversificación de otras especies mediterráneas. La radiación en la sect. *Supinae*

parece haber sido consecuencia de procesos de diferenciación alopátrida en poco tiempo. En este sentido, los análisis filogeográficos (que reconstruyen procesos recientes) a partir de DNA plastidial de las subsecciones *Saxatile* y *Supinae* muestran que dentro de ambas subsecciones la diferenciación ha tenido lugar de forma geográfica (Manuscrito 3, Blanco-Pastor & Vargas 2013), y que dicha diferenciación ha debido ocurrir de forma rápida (Manuscritos 4 y 5, Blanco-Pastor *et al.* 2013; Blanco-Pastor *et al.* ***submitted***). Además de la especiación alopátrida pura, otros tipos de especiación como la especiación híbrida o el refuerzo de la especiación mediado por polinizadores deben de haber jugado un papel importante en la generación de diversidad tanto en la sect. *Supinae* como en el conjunto de las angiospermas durante el Cuaternario.

Especiación híbrida

En eventos de hibridación, las barreras postcigóticas pueden dar lugar a especiación simpátrida mediante la incompatibilidad con los progenitores por reorganización en cromosomas o genes (Rieseberg 1997; Rieseberg & Willis 2007). En *Linaria* sect. *Supinae* los eventos de especiación híbrida homoploide han debido ser muy recurrentes tal y como ha mostrado el análisis de los factores responsables de la incongruencia filogenética mediante simulaciones de coalescencia (Manuscrito 2, Blanco-Pastor *et al.* 2012). En las angiospermas, los eventos de especiación híbrida homoploide son menos comunes que los eventos de especiación poliploide. Esto sucede porque el *fitness* de los híbridos de primera generación es normalmente muy reducido debido a las incompatibilidades genéticas. También porque debido a las débiles barreras reproductivas entre los híbridos y los progenitores se pueden producir episodios de retrocruzamiento (*back-crossing*) (Rieseberg & Willis 2007). Sin embargo, la especiación híbrida homoploide debe ser un proceso más común de lo pensado hasta ahora. La existencia de pocos casos descritos se debe en gran medida a la dificultad de su detección por falta de caracteres diagnósticos que permita una identificación indiscutible. Hay algunos ejemplos de especiación híbrida homoploide en la literatura (revisados en Gross & Rieseberg 2005), que reflejan la importancia de la selección ecológica para el éxito de los genotipos híbridos. En un futuro cercano, es altamente probable que sean descubiertas muchas más especies con origen híbrido mediante la aplicación de herramientas genómicas.

Investigaciones sobre zonas híbridas sugieren que, en el nicho de los progenitores, la mayoría de genotipos híbridos tienen menor *fitness* que las especies de las que se originan (Barton & Hewitt 1985), pero que ciertos genotipos híbridos pueden estar mejor adaptados que ambos progenitores a un tercer nicho (Grant 1981; Rieseberg 1997; Lexer *et al.* 2003; Rieseberg & Willis 2007). Al igual que

otras formas de especiación simpátrida, la especiación híbrida homoploide requiere cierto grado de separación de nicho entre las poblaciones progenitoras y la especie incipiente. De esta forma, la nueva especie híbrida generará novedades funcionales que le permitirían ocupar un nicho vacío (Stebbins 1959; Lewontin & Birch 1966; Templeton 1981; Arnold & Hodges 1995; Barton 2001; Lexer *et al.* 2003). Cambios ambientales que promueven el contacto secundario entre especies suelen dar lugar a nuevos hábitats (*hybrid habitats*, Templeton 1981). Las glaciaciones del Cuaternario constituyeron unas condiciones idóneas para el contacto secundario de especies próximas generadas en alopatría. En casos en los que el contacto secundario es recurrente se pueden generar radiaciones adaptativas estables (Anderson & Stebbins 1954; Seehausen 2004), proceso que podría ocurrir de forma extraordinariamente rápida (e.g. 10-60 generaciones, Ungerer *et al.* 1998; 10-500 generaciones, Buerkle *et al.* 2000). Después de que una radiación en alopatría haya progresado a una etapa en la que se hayan formado las nuevas especies, un contacto secundario promovido por el modelo de contracción-expansión consecuencia de las oscilaciones climáticas podría facilitar la hibridación y nueva diversificación. Sucesivos ciclos de especiación alopátrida y especiación híbrida habrían podido contrarrestar en gran medida los efectos de las extinciones durante los cambios climáticos. En la sect. *Supinae*, la especiación alopátrida promovida por la diferenciación geográfica durante los ciclos glaciales-interglaciales (Manuscrito 3, Blanco-Pastor & Vargas 2013) ha debido de venir acompañada de contacto secundario entre especies bien diferenciadas y generación de especies por hibridación homoploide (Manuscrito 2, Blanco-Pastor *et al.* 2012). Estos procesos repetidos a lo largo de los diferentes ciclos climáticos ha debido constituir el escenario para la diversificación de la sect. *Supinae,* así como para la generación de especies en otras radiaciones con origen reciente (e.g. Fiz-Palacios *et al.* 2010; Valente *et al.* 2010; Wilson & Hudson 2011).

Especiación mediada por polinizadores

El hipotético papel que habrían tenido los agentes polinizadores (especialmente los insectos) en la diversificación y en la evolución de la forma floral ha sido de especial interés para biólogos evolucionistas (Darwin 1877; Stebbins 1970; Kay & Sargent 2009). La divergencia adaptativa en respuesta a factores ecológicos como los polinizadores puede dar lugar a la evolución de barreras precigóticas y por lo tanto contribuye a la especiación (Rieseberg & Willis 2007). La peculiar forma y los atractivos colores de la flor personada de *Linaria* y otras Antirrhineas parecen claras adaptaciones a la polinización entomófila (Hill 1909; Kampny 1995; Endress 1999). En este sentido, las especies de la subsect. *Supinae* son polinizadas principalmente por abejas grandes de lengua larga, y reciben una baja diversidad de polinizadores (Manuscrito 5, Blanco-Pastor *et al.* ***submitted***).

El análisis de los caracteres florales en este grupo ha mostrado que especies especializadas a la polinización por un reducido grupo de abejas ha diversificado de forma muy rápida (Manuscrito 5, Blanco-Pastor *et al.* ***submitted***).

Otras presiones selectivas diferentes a los polinizadores parecen configurar las principales barreras reproductivas causantes de la especiación (Grant 1949, 1981; Hodges & Arnold 1994; Kay & Sargent 2009; Valente *et al.* 2012; Armbruster *et al.* 2013). En concreto, el aislamiento reproductivo promovido por polinizadores no debe ser el mecanismo principal de generación de especies de plantas en la sect. *Supinae*, así como en otros grupos de angiospermas (Grant 1949, 1981, 1994; Hodges & Arnold 1994; Kay & Sargent 2009; Valente *et al.* 2012; Armbruster *et al.* 2013). En *Supinae* se observa una fuerte señal geográfica en la diferenciación genética, lo cual parece indicar que éste ha sido el principal factor de diferenciación (Manuscrito 3, Blanco-Pastor & Vargas 2013). Sin embargo, está ampliamente consensuado que los polinizadores contribuyen a la diferenciación por su efecto en el aislamiento reproductivo y eficiencia reproductiva. Este aislamiento podría ocurrir de dos formas: tanto por el cambio de polinizador en simpatría, como por la especialización floral que promueve un refuerzo del aislamiento reproductivo durante el contacto secundario (*Reinforcement model*) (Dobzhansky 1937; Grant 1949; van der Niet *et al.* 2006; Armbruster & Muchhala 2009). Ciertas características de la Península Ibérica deberían facilitar el contacto secundario y la especiación híbrida tal y como hemos comentado anteriormente. Sin embargo otro patrón de especiación alternativo podría ser el refuerzo del aislamiento reproductivo durante el contacto secundario *(Reinforcement model*). Se sabe que la especiación alopátrida junto con refuerzo del aislamiento reproductivo constituye una forma de especiación mucho más rápida que la especiación alopátrida pura (Coyne & Orr 2004). De hecho, modelos muestran que el refuerzo puede completar la especiación en el orden de cientos o miles de generaciones tras el contacto secundario (Liou & Price 1994; Kelly & Noor 1996). En la sect. *Supinae* el modelo de refuerzo parece el más apropiado para explicar la generación rápida de especies con flores muy especializadas (Manuscrito 5, Blanco-Pastor *et al.* ***submitted***). La especialización floral mediante la restricción de acceso al néctar para ciertos polinizadores ha debido constituir un mecanismo de aislamiento reproductivo precigótico y de refuerzo de la especiación. En éste sentido, Grant (1950) ya consideró que cabría esperar una formación de especies de forma más rápida en linajes especializados a la polinización por abejas que en otros linajes polinizados por otro tipo de insectos menos constantes. Este efecto deberá ser aún más importante en especies polinizadas principalmente por un solo tipo de abeja, tal y como ocurre en la sect. *Supinae* (Manuscrito 5, Blanco-Pastor *et al.* ***submitted***).

Se ha argumentado que es difícil distinguir entre el modelo de refuerzo (*Reinforcement model*) y desplazamiento del carácter reproductivo (*Character displacement model*) (Butlin 1989; van der Niet *et al*. 2006; Armbruster & Muchhala 2009) como factor de la diferenciación floral. Sin embargo, en el modelo de desplazamiento de carácter (*Character displacement model*) la riqueza de especies viene determinada por otros factores no relacionados con la polinización, y por tanto la especialización floral es tan solo un mecanismo de eficiencia en la transmisión de polen. Ya que este modelo requiere una ausencia de interfertilidad entre especies, parece que no es el más factible para grupos de especies de diferenciación durante el Cuaternario. Debido a que la capacidad de cruzamiento y la producción de individuos híbridos son relativamente comunes en los grupos de plantas de diversificación reciente.

DIFERENTES PATRONES EVOLUTIVOS EN *LINARIA*: COMPARACIÓN ENTRE LA SECT. *SUPINAE* Y LA SECT. *VERSICOLORES*

De las siete secciones reconocidas en la última revisión taxonómica completa de *Linaria* (Sutton 1988) (ocho si tenemos en cuenta la sect. *Lectoplectron*, Pennell 1935; Valdés 1970a; Fernández-Mazuecos *et al.* 2013b), dos de ellas han sido analizadas en profundidad en los últimos años: la sect. *Versicolores* (Fernández-Mazuecos & Vargas 2011; Fernández-Mazuecos 2012; Fernández-Mazuecos *et al.* 2013a; Fernández-Mazuecos & Vargas 2013) y la sect. *Supinae* (objeto de estudio de la presente tesis doctoral, Blanco-Pastor *et al.* 2012; Blanco-Pastor *et al.* 2013; Blanco-Pastor & Vargas 2013; Blanco-Pastor *et al.* ***submitted***). Resulta sorprendente la diferencia encontrada en ciertos patrones evolutivos de ambas secciones, teniendo en cuenta el parentesco cercano y un mismo marco espacial (oeste del Mediterráneo) y temporal (Cuaternario) de la diversificación.

ORIGEN Y PATRONES BIOGEOGRÁFICOS

La sect. *Versicolores* tiene un origen algo más antiguo que la sect. *Supinae*, ya que la primera se originó en el Mioceno mientras que la segunda es íntegramente del Cuaternario. De esta forma se ha asociado la aparición de las linarias bífidas (sect. *Versicolores*) con la aridificación de la región mediterránea durante el Cenozoico (Fernández-Mazuecos & Vargas 2011). A su vez, el origen de la sect. *Supinae* se ha asociado al establecimiento del clima Mediterráneo durante el Pleistoceno y las oscilaciones típicas de los ciclos glaciales (Manuscrito 2, Blanco-Pastor *et al.* 2012). Sin embargo es cierto que la mayor parte de la diversificación de ambas secciones ha ocurrido durante el Cuaternario, probablemente como consecuencia de la adaptación paralela a ambientes xéricos y cambiantes de este periodo.

Los análisis biogeográficos sugieren que el origen espacial de la sect. *Versicolores* sería la Península Ibérica (Fernández-Mazuecos & Vargas 2011) al igual que el de la sect. *Supinae* (Manuscrito 3, Blanco-Pastor & Vargas 2013). En la sect. *Versicolores* se sucedería un evento de dispersión hacia el norte de Africa durante el Messiniense (periodo durante el cual África y Europa estuvieron conectadas) y posteriormente varios eventos de dispersión de África a Europa durante el Cuaternario, cuando la apertura del estrecho de Gibraltar y el llenado de la cuenca mediterránea constituyeron una barrera física para la dispersión. Estas dispersiones transmediterráneas durante el Cuaternario también han sido inferidas en la sect. *Supinae.* Debido a que la sect. *Versicolores* carece de estructuras especializadas para dispersión a larga distancia (presenta semillas ápteras) a diferencia de la sect. *Supinae* (semillas aladas), parece que esta característica no ha sido

determinante para el éxito en la colonización, como también se ha visto mediante análisis específicos en la sección *Supinae* (Manuscrito 3, Blanco-Pastor & Vargas 2013).

EVOLUCIÓN DE LA FORMA FLORAL

Los análisis de la forma de la corola han mostrado especialización en la sect. *Versicolores* mediante cambios recurrentes en la anchura del tubo floral. Estos cambios sugieren la existencia de unas presiones selectivas similares, probablemente asociadas a la restricción del acceso al néctar por parte de ciertos polinizadores y a la polinización por insectos capaces de transportar polen en la probóscide, entre los que se encuentran abejas del género *Antophora* (Fernández-Mazuecos *et al.* 2013a). Curiosamente en la sect. *Supinae* un tipo distinto de cambio morfológico de carácter restrictivo parece haber ocurrido en repetidas ocasiones. En este caso se han encontrado diferencias en la forma de la flor asociadas a un estrechamiento del espolón (no del tubo de la corola). Dicho cambio morfológico parece estar relacionado también con la especialización a ciertas abejas del género *Antophora* (Manuscrito 5, Blanco-Pastor *et al.* ***submitted***). Por lo tanto, parece ser que las especies de ambas secciones han seguido distintos patrones de diferenciación floral frente a unas mismas presiones selectivas relacionadas con la polinización.

EFECTO DE LAS GLACIACIONES EN ESPECIES MONTANAS

Se ha observado que los ciclos glaciales-interglaciales han afectado de forma diferente a la distribución en dos especies montanas: *L. elegans* (sect. *Versicolores*, 100-2400 m) y *L. glacialis* (sect. *Supinae*, 2700-3400 m). Análisis moleculares y de modelización de la distribución (modelo paleoclimático CCSM) indican que el último periodo glacial afectó de forma considerable la distribución de *L. elegans*, ya que esta especie habría quedado confinada a dos refugios glaciales en el noroeste peninsular asociados a un clima oceánico. A lo largo del presente período interglacial *L. elegans* habría aumentado considerablemente su área de distribución ocupando un anillo de montañas en la mitad norte de la Peninsula Ibérica (Región Cantábrica, Sistema Central y Sistema Ibérico). Inesperadamente, a partir de datos moleculares y de modelización de la distribución, se ha observado que la especie *L. glacialis* (endemismo de las cumbres de Sierra Nevada) ha sido mucho menos afectada por los ciclos climáticos del Cuaternario. De esta forma se observa que el área de distribución y la diversidad genética a lo largo de los últimos ciclos glaciales-interglaciales han variado muy poco en esta especie. La diferencia en los patrones de distribución durante el

Cuaternario observados en estas dos especies se explica por el limitado efecto que tuvieron las glaciaciones en el sur de la Península Ibérica si lo comparamos con áreas más septentrionales como las de *L. elegans*. Las montañas del sur peninsular constituyeron en sí refugios glaciales (Hewitt 1996; Comes & Kadereit 1998; Taberlet *et al.* 1998; Lobo *et al.* 2001; Médail & Diadema 2009). En particular, Sierra Nevada representa el límite sur de la influencia glacial en Europa debido a que estuvo cubierta de hielo solo parcialmente y por encima de c. 2500 m (Gómez-Ortiz *et al.* 1996), hecho que favoreció la persistencia de las especies en los distintos cinturones altitudinales.

COMENTARIO FINAL

Mediante el análisis de la historia evolutiva de *Linaria* sect. *Supinae* a distintos niveles taxonómicos y utilizando técnicas filogenéticas, filogeográficas y ecológicas, se ha observado que durante el período Cuaternario, un conjunto de presiones selectivas similares han dado lugar a distintos patrones evolutivos que incluyen: diversificación rápida vs. estasis evolutiva, endemicidad vs. expansión de la distribución y reducción del tamaño floral vs. especialización a ciertos polinizadores. Estos patrones evolutivos han estado marcados en gran medida por los sistemas reproductivos, el marco geográfico de diferenciación, las interacciones bióticas y el escenario climático. En este sentido, se ha podido demostrar que los biólogos evolucionistas de plantas clásicos, como Stebbins (1957, 1959, 1970; 1974), Grant (1958, 1981) o Baker (1955, 1959a, 1967), estuvieron acertados al interpretar que las plantas autógamas tendrían una gran capacidad de supervivencia a corto plazo pero una menor flexibilidad a largo plazo, más característica de plantas alógamas. Gracias al excepcional desarrollo analítico de los últimos tiempos, hoy en día tenemos una visión más desarrollada, no solo de los patrones microevolutivos sino también de patrones macroevolutivos. Esto nos permite establecer las relaciones entre el modo de reproducción y la supervivencia de las poblaciones y también la relación entre los sistemas reproductivos y las tasas de diversificación. Esta memoria de tesis doctoral muestra la diversidad de patrones evolutivos en *Linaria* sect. *Supinae*, que representa un excepcional grupo modelo para el estudio de la evolución de las plantas con flor durante el Cuaternario.

REFERENCIAS

Albaladejo RG, Aguilar JF, Aparicio A, Feliner GN (2005) Contrasting nuclear-plastidial phylogenetic patterns in the recently diverged Iberian *Phlomis crinita* and *P. lychnitis* lineages (Lamiaceae). *Taxon* **54**, 987-998.

Anderson E, Stebbins GL, Jr. (1954) Hybridization as an Evolutionary Stimulus. *Evolution* **8**, 378-388.

Andrew RL, Ostevik KL, Ebert DP, Rieseberg LH (2012) Adaptation with gene flow across the landscape in a dune sunflower. *Molecular Ecology* **21**, 2078-2091.

Armbruster WS, Muchhala N (2009) Associations between floral specialization and species diversity: cause, effect, or correlation? *Evolutionary Ecology* **23**, 159-179.

Armbruster WS, Shi X-Q, Huang S-Q (2013) Do specialized flowers promote reproductive isolation? Realized pollination accuracy of three sympatric *Pedicularis* species. *Annals of Botany*, doi: 10.1093/aob/mct1187.

Arnold ML, Hodges SA (1995) Are natural hybrids fit or unfit relative to their parents? *Trends in Ecology & Evolution* **10**, 67-71.

Baker HG (1953) Race formation and reproductive method in flowering plants **7**, 114-145.

Baker HG (1955) Self-Compatibility and Establishment After 'Long-Distance' Dispersal. *Evolution* **9**, 347-349.

Baker HG (1959a) The Contribution of Autecological and Genecological Studies to Our Knowledge of the Past Migrations of Plants. *The American Naturalist* **93**, 255-272.

Baker HG (1959b) Reproductive methods as factors in speciation in flowering plants. *Cold Spring Harbor Symposia on Quantitative Biology* **24**, 177-191.

Baker HG (1965) Characteristics and modes of origin of weeds. In: *The genetics of colonizing species.* (eds. Baker H, Stebbins G), pp. 147-168. Academic Press New York, New York.

Baker HG (1967) Support for Baker's Law-As a Rule. *Evolution* **21**, 853-856.

Barres L, Vilatersana R, Molero J, Susanna A, Galbany-Casals M (2011) Molecular phylogeny of *Euphorbia* subg. *Esula* sect. *Aphyllis* (Euphorbiaceae) inferred from nrDNA and cpDNA markers with biogeographic insights. *Taxon* **60**, 705-720.

Barton NH (2001) The role of hybridization in evolution. *Molecular Ecology* **10**, 551-568.

Barton NH, Hewitt G (1985) Analysis of hybrid zones. *Annual Review of Ecology and Systematics* **16**, 113-148.

Blanco-Pastor JL, Fernández-Mazuecos M, Vargas P (2013) Past and future demographic dynamics of alpine species: limited genetic consequences despite dramatic range contraction in a plant from the Spanish Sierra Nevada. *Molecular Ecology* **22**, 4177-4195.

Blanco-Pastor JL, Ornosa C, Romero D, *et al.* (***submitted***) Bee morphotypes explain floral variation in a radiation of *Linaria* species. *Journal of evolutionary biology*.

Blanco-Pastor JL, Vargas P (2013) Autecological traits determined two evolutionary strategies in Mediterranean plants during the Quaternary: low differentiation and range expansion versus geographical speciation in *Linaria*. *Molecular Ecology* **22**, 5651–5668.

Blanco-Pastor JL, Vargas P, Pfeil BE (2012) Coalescent Simulations Reveal Hybridization and Incomplete Lineage Sorting in Mediterranean *Linaria*. *Plos One* **7**, e39089.

Boissier E (1879) *Flora orientalis. Vol. 4*, Geneva and Basel.

Brennan AC, Harris SA, Hiscock SJ (2005) Modes and rates of selfing and associated inbreeding depression in the self-incompatible plant *Senecio squalidus* (Asteraceae): a successful colonizing species in the British Isles. *New Phytologist* **168**, 475-486.

Buerkle CA, Morris RJ, Asmussen MA, Rieseberg LH (2000) The likelihood of homoploid hybrid speciation. *Heredity* **84**, 441-451.

Busch JW, Joly S, Schoen DJ (2011) Demographic Signatures Accompanying the Evolution of Selfing in *Leavenworthia alabamica*. *Molecular Biology and Evolution* **28**, 1717-1729.

Butlin R (1989) Reinforcement of premating isolation. In: *Speciation and its Consequences* (eds. Otte D, Endler JA), pp. 158-179, Sinauer, Sunderland, MA.

Carlquist S (1966) The Biota of Long-Distance Dispersal. IV. Genetic Systems in the Floras of Oceanic Islands. *Evolution* **20**, 433-455.

Charlesworth D, Pannell J (2001) Mating systems and population genetic structure in the light of coalescent theory. In: *Integrating Ecology and Evolution in a Spatial Context* (eds. Silvertown J, Antonovics J), pp. 73-96. British Ecological Society.

Comes HP, Abbott RJ (2001) Molecular phylogeography, reticulation, and lineage sorting in mediterranean *Senecio* sect. *Senecio* (Asteraceae). *Evolution* **55**, 1943-1962.

Comes HP, Kadereit JW (1998) The effect of Quaternary climatic changes on plant distribution and evolution. *Trends in Plant Science* **3**, 432-438.

Coyne JA, Orr HA (2004) *Speciation* Sinauer Associates, Sunderland, Massachusetts U.S.A. .

Darlington CD (1958) *The Evolution of Genetic Systems* Cambridge University Press, Cambridge, England.

Darwin C (1877) *The different forms of flowers on plants of the same species* John Murray, London. UK.

Dobzhansky T (1937) *Genetics and the Origin of Species* Columbia University Press, New York.

Drummond AJ, Ho SYW, Phillips MJ, Rambaut A (2006) Relaxed Phylogenetics and Dating with Confidence. *PLoS Biol* **4**, e88.

Endler JA (1973) Gene Flow and Population Differentiation: Studies of clines suggest that differentiation along environmental gradients may be independent of gene flow. *Science* **179**, 243-250.

Endress PK (1999) Symmetry in Flowers: Diversity and Evolution. *International Journal of Plant Sciences* **160**, S3-S23.

Ennos RA, French GC, Hollingsworth PM (2005) Conserving taxonomic complexity. *Trends in Ecology & Evolution* **20**, 164-168.

Feliner GN (2011) Southern European glacial refugia: A tale of tales. *Taxon* **60**, 365-372.

Fernández-Mazuecos M (2012) *Evolución en el género Linaria: biogeografía, cambio morfológico y sistemática de Linaria sect. Versicolores*, Departamento de Biología. Facultad de Ciencias (Universidad Autónoma de Madrid).

Fernández-Mazuecos M, Blanco-Pastor JL, Gómez JM, Vargas P (2013a) Corolla morphology influences diversification rates in bifid toadflaxes (*Linaria* sect. *Versicolores*). *Annals of Botany* **112**, 1705-1722.

Fernández-Mazuecos M, Blanco-Pastor JL, Vargas P (2013b) A Phylogeny of Toadflaxes (*Linaria* Mill.) Based on Nuclear Internal Transcribed Spacer Sequences: Systematic and Evolutionary Consequences. *International Journal of Plant Sciences* **174**, 234-249.

Fernández-Mazuecos M, Vargas P (2011) Historical isolation *versus* recent long-distance connections between Europe and Africa in bifid toadflaxes (*Linaria* sect. *Versicolores*) *Plos One* **6**, e22234.

Fernández-Mazuecos M, Vargas P (2013) Congruence between distribution modelling and phylogeographical analyses reveals Quaternary survival of a toadflax species (*Linaria elegans*) in oceanic climate areas of a mountain ring range. *New Phytologist* **198**, 1274-1289.

Fishman L, Stratton DA (2004) The genetics of floral divergence and postzygotic barriers between outcrossing and selfing populations of Arenaria uniflora (Caryophyllaceae). *Evolution* **58**, 296-307.

Fiz-Palacios O, Vargas P, Vila R, Papadopulos AST, Aldasoro JJ (2010) The uneven phylogeny and biogeography of *Erodium* (Geraniaceae): radiations in the Mediterranean and recent recurrent intercontinental colonization. *Annals of Botany* **106**, 871-884.

Frajman B, Eggens F, Oxelman B (2009) Hybrid origins and homoploid reticulate evolution within *Heliosperma* (Sileneae, Caryophyllaceae)-A multigene phylogenetic approach with relative dating. *Systematic Biology* **58**, 328-345.

Gabriel W, Lynch M, Burger R (1993) Muller's ratchet and mutational meltdowns. *Evolution* **47**, 1744-1757.

Goldberg EE, Igić B (2012) Tempo and mode in plant breeding system evolution. *Evolution* **66**, 3701-3709.

Goldberg EE, Kohn JR, Lande R, *et al.* (2010) Species Selection Maintains Self-Incompatibility. *Science* **330**, 493-495.

Gómez-Ortiz A, Schulte L, Salvador-Franch F (1996) Contribución al conocimiento de la deglaciación reciente y morfología asociada del Corral del Veleta (Sierra Nevada). *Cadernos do Laboratorio Xeolóxico de Laxe* **21**, 543-558.

Grant V (1949) Pollination Systems as Isolating Mechanisms in Angiosperms. *Evolution* **3**, 82-97.

Grant V (1950) The flower constancy of bees. *The Botanical Review* **16**, 379-398.

Grant V (1958) The Regulation of Recombination in Plants. *Cold Spring Harbor Symposia on Quantitative Biology* **23**, 337-363.

Grant V (1981) *Plant Speciation*, 2nd edn. Columbia University Press, New York.

Grant V (1994) Modes and origins of mechanical and ethological isolation in angiosperms. *Proceedings of the National Academy of Sciences* **91**, 3-10.

Grantham T (2007) Is macroevolution more than successive rounds of microevolution? *Palaeontology* **50**, 75-85.

Gross B, Rieseberg L (2005) The ecological genetics of homoploid hybrid speciation. *Journal of Heredity* **96**, 241-252.

Grossenbacher DL, Whittall JB (2011) Increased floral divergence in sympatric monkeyflowers. *Evolution* **65**, 2712-2718.

Guo YL, Bechsgaard JS, Slotte T, *et al.* (2009) Recent speciation of *Capsella rubella* from *Capsella grandiflora*, associated with loss of self-incompatibility and an extreme bottleneck. *Proceedings of the National Academy of Sciences* **106**, 5246-5251.

Guo YP, Ehrendorfer F, Samuel R (2004) Phylogeny and systematics of *Achillea* (Asteraceae-Anthemideae) inferred from nrITS and plastid trnL-F DNA sequences. *Taxon* **53**, 657-672.

Hamrick JL, Godt MJW (1996) Effects of Life History Traits on Genetic Diversity in Plant Species. *Philosophical Transactions: Biological Sciences* **351**, 1291-1298.

Hewitt G (2001) Speciation, hybrid zones and phylogeography- or seeing genes in space and time. *Molecular Ecology* **10**, 537-549.

Hewitt GM (1996) Some genetic consequences of ice ages, and their role in divergence and speciation. *Biological Journal of the Linnean Society* **58**, 247-276.

Hill EJ (1909) Pollination in *Linaria* with special reference to cleistogamy. *Botanical Gazette* **47**, 454-466.

Hodges SA, Arnold ML (1994) Floral and ecological isolation between *Aquilegia formosa* and *Aquilegia pubescens*. *Proceedings of the National Academy of Sciences* **91**, 2493-2496.

Holsinger KE (2000) Reproductive systems and evolution in vascular plants. *Proceedings of the National Academy of Sciences* **97**, 7037-7042.

Igic B, Lande R, Kohn J (2008) Loss of Self-Incompatibility and Its Evolutionary Consequences. *International Journal of Plant Sciences* **169**, 93-104.

Jablonski D (2007) Scale and hierarchy in macroevolution. *Palaeontology* **50**, 87-109.

Jain S (1976) The evolution of inbreeding in plants. *Annual Review of Ecology and Systematics* **7**, 469-495.

Kampny CM (1995) Pollination and flower diversity in Scrophulariaceae. *The Botanical Review* **61**, 350-366.

Kay KM, Sargent RD (2009) The role of animal pollination in plant speciation: integrating ecology, geography, and genetics. *Annual Review of Ecology, Evolution, and Systematics* **40**, 637-656.

Kelly JK, Noor MAF (1996) Speciation by Reinforcement: A Model Derived from Studies of Drosophila. *Genetics* **143**, 1485-1497.

Lange J (1870) Scrophulariaceae. In: *Prodromus florae Hispanicae* (eds. Willkomm M, Lange J), pp. 546-620, Stuttgart.

Lewontin RC, Birch LC (1966) Hybridization as a Source of Variation for Adaptation to New Environments. *Evolution* **20**, 315-336.

Lexer C, Welch ME, Raymond O, Rieseberg LH (2003) The origin of ecological divergence in Helianthus paradoxus (Asteraceae): selection on transgressive characters in a novel hybrid habitat. *Evolution* **57**, 1989-2000.

Liou LW, Price TD (1994) Speciation by Reinforcement of Premating Isolation. *Evolution* **48**, 1451-1459.

Lledó MD, Crespo MB, Fay MF, Chase MW (2005) Molecular phylogenetics of Limonium and related genera (Plumbaginaceae): biogeographical and systematic implications. *American Journal of Botany* **92**, 1189-1198.

Lo Presti RM, Oberprieler C (2011) The central Mediterranean as a phytodiversity hotchpotch: phylogeographical patterns of the *Anthemis secundiramea* group (Compositae, Anthemideae) across the Sicilian Channel. *Journal of Biogeography* **38**, 1109-1124.

Lobo JM, Castro I, Moreno JC (2001) Spatial and environmental determinants of vascular plant species richness distribution in the Iberian Peninsula and Balearic Islands. *Biological Journal of the Linnean Society* **73**, 233-253.

Lowry E, Lester SE (2006) The biogeography of plant reproduction: Potential determinants of species' range sizes. *Journal of Biogeography* **33**, 1975-1982.

Mansion G, Zeltner L, Bretagnolle F (2005) Phylogenetic patterns and polyploid evolution within the Mediterranean genus *Centaurium* (Gentianaceae - Chironieae). *Taxon* **54**, 931-950.

Martin NH, Willis JH (2007) Ecological divergence associated with mating system causes nearly complete reproductive isolation between sympatric *Mimulus* species. *Evolution* **61**, 68-82.

Maureira-Butler IJ, Pfeil BE, Muangprom A, Osborn TC, Doyle JJ (2008) The reticulate history of *Medicago* (Fabaceae). *Systematic Biology* **57**, 466-482.

Mayr E (1965) Summary. In: *The genetics of colonizing species.* (eds. Baker H, Stebbins G), pp. 429-447. Academic Press New York, New York.

Médail F, Diadema K (2009) Glacial refugia influence plant diversity patterns in the Mediterranean Basin. *Journal of Biogeography* **36**, 1333-1345.

Morison R (1680) *Plantarum historiae universalis Oxoniensis. Vol. 2*, Oxford.

Pennell FW (1935) The Scrophulariaceae of eastern temperate North America. *The Academy of Natural Sciences of Philadelphia Monographs* **1**, 1-650.

Presti RML, Oppolzer S, Oberprieler C (2010) A molecular phylogeny and a revised classification of the mediterranean genus *Anthemis* s.l. (Compositae, Anthemideae) based on three molecular markers and micromorphological characters. *Taxon* **59**, 1441-1456.

Rambuda TD, Johnson SD (2004) Breeding systems of invasive alien plants in South Africa: does Baker's rule apply? *Diversity and Distributions* **10**, 409-416.

Randle AM, Slyder JB, Kalisz S (2009) Can differences in autonomous selfing ability explain differences in range size among sister-taxa pairs of *Collinsia* (Plantaginaceae)? An extension of Baker's Law. *New Phytologist* **183**, 618-629.

Rieseberg LH (1997) Hybrid origins of plant species. *Annual Review of Ecology and Systematics* **28**, 359-389.

Rieseberg LH, Soltis D (1991) Phylogenetic consequences of cytoplasmic gene flow in plants. *Evolutionary Trends in Plants* **5**.

Rieseberg LH, Willis JH (2007) Plant Speciation. *Science* **317**, 910-914.

Rodríguez-Sánchez F, Pérez-Barrales R, Ojeda F, Vargas P, Arroyo J (2008) The Strait of Gibraltar as a melting pot for plant biodiversity. *Quaternary Science Reviews* **27**, 2100-2117.

Sánchez-Lafuente A (2007) Corolla herbivory, pollination success and fruit predation in complex flowers: An experimental study with *Linaria lilacina* (Scrophulariaceae). *Annals of Botany* **99**, 355-364.

Sánchez-Lafuente AM, Rodríguez-Gironés MA, Parra R (2011) Interaction frequency and per-interaction effects as predictors of total effects in plant–pollinator mutualisms: a case study with the self-incompatible herb *Linaria lilacina*. *Oecologia* **168**, 153-165.

Seehausen O (2004) Hybridization and adaptive radiation. *Trends in Ecology and Evolution* **19**, 198-207.

Segarra-Moragues JG, Mateu-Andres I (2007) Levels of allozyme diversity in closely related toadflaxes (*Linaria*, Plantaginaceae) and their correspondence with the breeding systems of the species. *Conservation Genetics* **8**, 373-383.

Soltis PS, Soltis DE (2009) The Role of Hybridization in Plant Speciation. *Annual Review of Plant Biology* **60**, 561-588.

Stebbins GL (1957) Self Fertilization and Population Variability in the Higher Plants. *The American Naturalist* **91**, 337-354.

Stebbins GL (1959) The Role of Hybridization in Evolution. *Proceedings of the American Philosophical Society* **103**, 231-251.

Stebbins GL (1970) Adaptive Radiation of Reproductive Characteristics in Angiosperms, I: Pollination Mechanisms. *Annual Review of Ecology and Systematics* **1**, 307-326.

Stebbins GL (1974) *Flowering plants: evolution above the species level* Belknap Press of Harvard University Press, Cambridge, Massachusetts.

Suc JP (1984) Origin and evolution of the Mediterranean vegetation and climate in Europe. *Nature* **307**, 429-432.

Sutton DA (1988) *A revision of the tribe Antirrhineae* Oxford University Press, Oxford.

Taberlet P, Fumagalli L, Wust-Saucy AG, Cosson JF (1998) Comparative phylogeography and postglacial colonization routes in Europe. *Molecular Ecology* **7**, 453-464.

Templeton AR (1981) Mechanisms of speciation--a population genetic approach. *Annual Review of Ecology and Systematics* **12**, 23-48.

Thoday J, Gibson J (1962) Isolation by disruptive selection. *Nature* **193**, 1164-1166.

Ungerer MC, Baird SJE, Pan J, Rieseberg LH (1998) Rapid hybrid speciation in wild sunflowers. *Proceedings of the National Academy of Sciences* **95**, 11757-11762.

Valcárcel V, Vargas P, Feliner GN (2006) Phylogenetic and phylogeographic analysis of the western Mediterranean *Arenaria* section *Plinthine* (Caryophyllaceae) based on nuclear, plastid, and morphological markers. *Taxon* **55**, 297-312.

Valdés B (1970a) Revisión de las especies europeas de *Linaria* con semillas aladas. *Anales de la Universidad Hispalense* **7**, 1-288.

Valdés B (1970b) Taxonomía experimental del género *Linaria* V. Hibridacion interespecifica. *Acta Phytotaxonomica Barcinonensia* **4**.

Valente LM, Manning JC, Goldblatt P, Vargas P (2012) Did pollination shifts drive diversification in southern African *Gladiolus*? Evaluating the model of pollinator-driven speciation. *The American Naturalist* **180**, 83-98.

Valente LM, Savolainen V, Vargas P (2010) Unparalleled rates of species diversification in Europe. *Proceedings of the Royal Society B: Biological Sciences* **277**, 1489-1496.

van der Niet T, Johnson SD, Linder HP (2006) Macroevolutionary data suggest a role for reinforcement in pollination system shifts. *Evolution* **60**, 1596-1601.

Vargas P, Valente LM, Blanco-Pastor JL, *et al.* (*in press*) Testing the biogeographical congruence of palaeofloras using molecular phylogenetics: snapdragons and the Madrean–Tethyan flora. *Journal of Biogeography* **Accepted**.

Viano J (1978a) Les linaires à graines aptères du bassin méditerranéen occidental. 1. *Linaria* sect. *Versicolores*. *Candollea* **33**, 33-88.

Viano J (1978b) Les linaires à graines aptères du bassin méditerranéen occidental. 2. *Linaria* sect. *Elegantes*, *Bipunctatae*, *Diffusae*, *Speciosae*, *Repentes*. *Candollea* **33**, 209-267.

Webb T, Bartlein P (1992) Global changes during the last 3 million years: climatic controls and biotic responses. *Annual Review of Ecology and Systematics* **23**, 141-173.

Wettstein R (1895) Scrophulariaceae. In: *Die natürligen pflanzenfamilien* (eds. Engler A, Prantl K), pp. 39-107, Lepzig.

Wilson Y, Hudson A (2011) The evolutionary history of *Antirrhinum* suggests that ancestral phenotype combinations survived repeated hybridizations. *Plant Journal* **66**, 1032-1043.

CONCLUSIONES

1. In our ITS phylogeny, all sampled species of the two toadflax genera (*Linaria* and *Nuttallanthus*) formed a strongly supported monophyletic group. The genus *Linaria* was recovered as a paraphyletic group, with the three sampled species of *Nuttallanthus* nested within it.
2. The hypothesis of a basal dichotomy in *Linaria* in which species with wingless and winged seeds constitute two natural sister lineages was clearly rejected.
3. Several shifts in seed morphology (wingless seeds, winged seeds, and seeds with a marginal ridge) appear to have occurred in the course of *Linaria* evolution.
4. Some sections of *Linaria* that are well defined by distinct morphological traits (sects. *Macrocentrum*, *Pelisserianae*, and *Versicolores*) were found to be monophyletic. Whereas, the remaining sections (*Supinae*, *Linaria*, *Speciosae*, *Diffusae*) were not supported as monophyletic groups in the ITS phylogeny.
5. Monophyly of section *Supinae* was supported in the ITS phylogeny when *L. latifolia* was excluded.
6. Simulations under the coalescent following the method of Maureira-Butler *et al.* (Syst. Biol. 57, 2008) showed that with small and medium effective population sizes, incomplete lineage sorting alone could not explain the topological incongruence among gene trees of *Supinae*. These results indicated that hybridization may also account for gene tree inconsistency.
7. The *BEAST multilabelled species tree is a method that allows the possibility of observing multiple congruent placements for the same individual. This approach combines the ideals of utilizing data sets of hybrids while also appropriately accommodating incomplete lineage sorting.
8. A comparison between the multilabelled species tree of *Supinae* and a total evidence analysis (based on concatenation) revealed that the latter approach could give misleading evolutionary reconstructions in species groups affected by incomplete lineage sorting and hybridization.
9. The *Linaria* species tree recovered section *Supinae* as monophyletic, which was divided into three morphologically-based subclades consistent with life-form, corolla size and seed wing shape.
10. Diversification of *Supinae* lineages may have occurred during the climatic oscillations of the Quaternary, after the establishment of the Mediterranean climate regime.

11. Out of ten potential hybridization events detected, one may have occurred within the subsect. *Supinae*; three between subsect. *Supinae* and a lineage containing both subsect. *Saxatile* and subsect. *Arvenses* species; and six within the latter lineage. Hybridization signal was also supported by morphology and previously published experimental crosses.
12. Both, rapid allopatric differentiation and secondary contact may have been evolutionary processes of *Supinae.*
13. The ancestral range reconstruction analysis placed the diversification of *Supinae* lineages in the Iberian Peninsula.
14. During the same period of time three species (*L. arvensis*, *L. micrantha* and *L. simplex*) colonized territories over long distances throughout the Mediterranean basin and Europe, whereas 31 species remained restricted to the western Mediterranean region.
15. Biogeographic reconstruction supported the occurrence of at least four successful long-distance dispersal events across the Mediterranean Sea during the colonization of northern Africa by *L. arvensis*, *L. micrantha* and *L. simplex*.
16. Subsect. *Saxatilis* and subsect. *Supinae* plastid lineages differentiated geographically in narrow ranges of the Iberian Peninsula, which indicates limitations of most species to range expansion.
17. Phylogeographic analyses of subsect. *Arvenses* suggested the occurrence of long-distance dispersal events in two distinct areas of the Mediterranean basin: the Strait of Gibraltar and the central Mediterranean.
18. Analysis of correlated evolution did not support a significant association between range expansion ability and seed dispersal structures but detected a positive association with (i) the ability to tolerate a wide variety of substrates, (ii) lifespan and (iii) breeding system.
19. A combination of the three traits (reproductive assurance, short generation time and the ability to tolerate a wide variety of substrates), were required for the range expansion success of *Supinae* species over long distances.
20. Contrasting diversification rates in *Supinae* lineages are explained by unequal speciation rates, which are higher for allogamous species.
21. Flower sizes of *Supinae* subsect. *Supinae* species showed neither fit nor correlation with the sizes of their principal pollinators. In contrast, flower shape differences associated to pollinator morphotypes were observed.

22. Phylogenetic constraints may have not played an important role in shaping the floral morphology and pollinator's preferences in *Supinae* subsect. *Supinae*
23. Morphological variation in flowers seem to be linked to the restriction in the access of nectar reward by certain pollinators.
24. The most restrictive flowers of *Supinae* subsect. *Supinae* were found in populations mainly pollinated by *Anthophora* and *Xylocopa* bees. Morphological change towards more restrictive spurs in those flowers may have reduced visitation of other bees with shorter proboscis.
25. Flower specialization in subsect. *Supinae* may have reinforced pre-existing reproductive isolation among interfertile *Linaria* species originated in a radiation.
26. *L. glacialis* may have persisted on the Sierra Nevada summits during the late Quaternary climatic changes, while experiencing contraction–expansion processes. However, such processes seem to have been limited, if compared with the severe range contractions of future projections.
27. In the last millennium, climatic oscillations moderately affected the demographic trends of *L. glacialis*. A slight population size decline was initiated in the early stages of the last Holocene warm period (Medieval Warm Period), which was followed by a recovery in size and a subsequent expansion that coincided with the following cold stage (Little Ice Age).
28. *L. glacialis* currently maintains an unexpectedly high degree of genetic diversity given the estimated effective population size (~30,000/~15,000 ind.) and the presumed census size (~10,000 ind.)
29. Distribution models predicted severe range contractions of *L. glacialis* in the near future, which illustrates the extreme vulnerability of this species to temperature rising.
30. Future climatic conditions will not lead to a progressive genetic loss, but can lead to the extinction of *L. glacialis* with instantaneous loss of the entire gene pool in a very short time period.
31. The evolutionary change to a mixed mating system of *L. glacialis* may have promoted survival and gene flow in the extreme alpine environment of Sierra Nevada summits.

APÉNDICES:

OTRAS APORTACIONES CIENTÍFICAS DERIVADAS DE LA TESIS DOCTORAL

Durante el desarrollo de la presente tesis doctoral, el autor ha participado en la elaboración de tres publicaciones científicas adicionales. Su contribución ha sido la siguiente:

Apéndice 1- Vargas, P., Valente, L.M., Blanco-Pastor, J.L., Liberal, I., Guzmán, B., Cano, E., Forrest, A. and Fernández-. Mazuecos, M. (2013) Testing biogeographic congruence of palaeofloras using molecular phylogenetics: snapdragons and the Madrean-Tethyan flora. **Journal of Biogeography**, accepted doi:10.1111/jbi.12253

Elaboración de análisis biogeográficos

Apéndice 2 - Fernández-Mazuecos, M., Blanco-Pastor, J.L., Gómez, J.M. & Vargas, P. (2013) Corolla morphology influences diversification rates in bifid toadflaxes (*Linaria* sect. *Versicolores*). **Annals of Botany**, 112 (9): 1705-1722

Participación en los análisis de diversificación

Apéndice 3 - Vargas, P., Ornosa, C., Blanco-Pastor, J.L., Romero, D., Fernández-Mazuecos, M., Rodríguez-Gironés, M.A. (2013). En búsqueda de áreas de diversidad genética en Sierra Nevada: análisis de plantas y abejas. En: Ramírez, L., Asensio, B. (eds.). Proyectos de investigación en parques nacionales: 2009-2012, pp. 123-142. Organismos Autónomo de Parques Nacionales. Madrid. ISBN 978-84-8014-853-5.

Participación en el muestreo, elaboración de análisis y figuras

Appendix 1

Vargas, P. *et al.* (2013), "Testing biogeographic congruence of palaeofloras using molecular phylogenetics: snapdragons and the Madrean-Tethyan flora"

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ORIGINAL ARTICLE

Testing the biogeographical congruence of palaeofloras using molecular phylogenetics: snapdragons and the Madrean–Tethyan flora

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ABSTRACT

Aim The biogeographical congruence hypothesis, that similar spatiotemporal patterns of geographical distribution exist across lineages, is revisited in this study, and biogeographical processes in presumed Madrean–Tethyan plants are investigated by employing phylogenetic analyses.

Location Mediterranean and Californian floristic regions.

Methods The snapdragons (tribe Antirrhineae, Plantaginaceae) are one of the plant groups that best illustrate disjunctions between the New World (14 genera) and the Old World (15 genera). A time-calibrated phylogeny (*ndh*F sequences) and ancestral-area reconstructions were used to test the hypothesis of biogeographical congruence. We estimated support for sister-group relationships together with the probability of temporal congruence of snapdragons and five additional angiosperm groups using a biogeographical approach based on Bayesian inference, parsimony and maximum-likelihood methods.

Results Synchronous divergences of four phylogenetically independent Mediterranean/Californian lineages within Antirrhineae were inferred for the Miocene. This result constitutes the first example of high biogeographical congruence within the same plant group. Analyses of five additional angiosperm groups previously considered exemplars of Madrean–Tethyan disjunctions revealed a total of 10 Mediterranean/Californian sister-group lineages, mostly with Miocene divergence times. In particular, our contrasting biogeographical analysis favoured a prevalent colonization process post-dating the separation of America and Eurasia (Eocene) for at least eight angiosperm lineages.

Main conclusions Explicit testing of the Madrean–Tethyan hypothesis did not support predominant vicariance for Mediterranean/Californian sister groups as previously proposed. Instead, eight Mediterranean/Californian sister-group lineages displayed a Miocene divergence, including considerable biogeographical congruence within Antirrhineae (four independent lineages) and Cistaceae (two lineages).

Keywords
Antirrhineae, Arbutoideae, California, *Cercis*, Cistaceae, disjunct distributions, Geraniaceae, Mediterranean floristic regions, *Platanus*, vicariance.

INTRODUCTION

Lineages with discontinuous distributions provide ideal systems in which to investigate biogeographical processes. The lure of understanding fragmented distributions has historically led biogeographers to recognize different categories of intercontinental disjunctions (Raven, 1972; Thorne, 1972). Two main hypotheses underlying disjunct distributions have historically been tested: vicariance (fragmentation of a widespread distribution due to the formation of an isolating barrier) and long-distance dispersal (overcoming of a preexisting barrier by propagules that give rise to disjunct

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populations) (Crisp *et al.*, 2011). Under the vicariance hypothesis, the formation of the barrier coincided with the origin of the disjunction, whereas in the dispersal hypothesis, the barrier is assumed to have pre-dated the disjunction (Morrone & Crisci, 1995; Crisp *et al.*, 2011). Historical biogeography (palaeobiogeography) is concerned not only with barriers and past distributions of particular species groups and their relatives, but also with range shifts and the divergence times of independent lineages.

One of the most intriguing and least studied biogeographical patterns is biogeographical congruence, in which not only do two or more lineages share the same geographical distribution, but this distribution also originated during the same time period (Cunningham & Collins, 1994). In other words, synchronous vicariance or dispersal events reflect shared historical processes. A comparable, but very different, pattern is biogeographical pseudocongruence, which occurs when the common geographical distribution was established at different times, and is thus potentially attributable to different biogeographical processes and conditions (Page, 1990; Cunningham & Collins, 1994; Wen, 1999; Donoghue & Moore, 2003). In order to distinguish between biogeographical congruence and pseudocongruence for lineages that share discontinuous distributions, a temporal framework is required to examine whether disjunctions occurred synchronously and hence may be the result of common processes (congruence), or asynchronously and thus might be attributable to different processes (pseudocongruence) (Donoghue & Moore, 2003).

Fossil occurrences assist in reconstructing distribution patterns over a period of time, but their fragmentary distribution results in many large temporal and spatial gaps. Molecular phylogenetics can provide a reliable spatiotemporal reconstruction of biogeographical events, especially when complemented by chorology, palaeontology and palaeoecology. Therefore, the use of sophisticated molecular phylogenetic methods (see Ronquist & Sanmartín, 2011) has led to detection of an increasing number of coincidental patterns of historical biogeography of animals and plants (Donoghue & Smith, 2004).

Some of the most-studied disjunctions are north temperate discontinuous distributions that include lineages distributed in Asia, Europe and North America (Raven, 1972). The Madrean–Tethyan hypothesis suggests that the formation of two floristic regions (Mediterranean and Californian) that have a summer-drought climate in about the same period of the year (May–September) may be the result of a vicariance process and persistence since the early Tertiary (Palaeogene) (Axelrod, 1975; Raven & Axelrod, 1978). Both the Atlantic Ocean and the summer-humid climate of eastern North America constitute significant present-day dispersal barriers between these two floristic regions. Taxa that occur in both regions have been considered to be representatives of the ancient (early Tertiary) Madrean–Tethyan flora (see Raven & Axelrod, 1978), which consisted of plants in summer-dry areas of the Madrean region (present Sierra Madre Occidental and California in south-western North America) and the Tethys region (the present Mediterranean basin) (see Takhtajan, 1986). By the later Palaeogene, sclerophyllous vegetation adapted to the expanding dry climate is thought to have formed a dominant, nearly continuous belt across North America and Eurasia (Axelrod, 1975). The taxonomic affinity between plants of the Mediterranean and Californian regions has been hypothesized to reveal a historical signal of ancient Madrean–Tethyan vicariance that has persisted in disjunct Mediterranean-like climates after the opening of the Atlantic Ocean in the Eocene (Thorne, 1972; Axelrod, 1975; Tiffney & Manchester, 2001).

The Madrean–Tethyan vicariance hypothesis has been challenged by a number of molecular phylogenetic studies, primarily in the last two decades (Fritsch, 1996; Manos & Donoghue, 2001). Wen & Ickert-Bond (2009) comprehensively reviewed molecular phylogenies addressing the current disjunction of Madrean–Tethyan plant groups. They reviewed phylogenetic relationships and divergence times between lineages of the two regions and concluded: (1) that vicariance appears to be unlikely given the roughly estimated recent divergence times (< 25 Ma); (2) that convergent evolution may have reinforced morphological and floristic similarity; and (3) that the predominant direction of migration/dispersal was from the Old World to the New World. However, they acknowledged that most of the 14 angiosperm study groups used for the analysis lacked fossil calibrations and an explicit methodology for estimation of divergence times. Preliminary dating results yielded a wide range of divergence times (Eocene to Pleistocene). Likewise, Kadereit & Baldwin (2012) reviewed the same plant groups, and additional data from other groups, and also found that lineages were connected by migrations at different times (but mostly in the Miocene). The question, however, remains as to whether a high degree of biogeographical congruence can be statistically supported for Madrean–Tethyan lineages (Crisp *et al.*, 2011). In particular, to what extent do coincident timings of colonization pre-date the (Eocene) North America–Europe split and therefore constitute a vicariance process of divergence?

It has been suggested that the tribe Antirrhineae (snapdragons) is one of the best candidates to test the biogeographical congruence hypothesis (hereafter BCH) (Raven & Axelrod, 1978), because at least three lineages in the tribe have been found to display a similar Madrean–Tethyan distribution pattern (Vargas *et al.*, 2004). The main objective of this study is therefore to test the spatiotemporal lineage divergence patterns of tribe Antirrhineae in order to account for the origin of the Madrean–Tethyan disjunction and to disentangle biogeographical congruence and pseudocongruence in snapdragons. In addition, to explicitly test the hypothesis of a vicariant origin of the Madrean–Tethyan palaeoflora, we reanalysed existing molecular data for five of the 28 angiosperm groups proposed by Raven & Axelrod (1978). Our working hypothesis is that there is biogeographical congruence within Antirrhineae and with the five additional Madrean–Tethyan angiosperm groups, owing to synchronous divergent events. Finally, we revisit the concept of

biogeographical congruence and propose essential conditions to be met by any group under the BCH.

MATERIALS AND METHODS

Testing the biogeographical congruence hypothesis

Phylogenetic inference can help provide the evidence required to test the BCH for the Madrean–Tethyan flora, specifically: (1) common ancestry of plants involved in the Old/New World disjunction as hypothesized based on taxonomy; (2) an unequivocal sister relationship between Mediterranean and Californian clades; (3) availability of appropriate fossil-based calibration points and estimates of molecular substitution rates for phylogenetic dating; (4) sharing of the same temporal framework of biogeographical events compatible with vicariance or dispersal; and (5) identification of two or more lineages displaying the same biogeographical disjunction and temporal pattern within or across plant groups. In this study, most recent common ancestors (MRCA) and sister-group relationships were inferred based on monophyly of DNA sequences. Molecular clock techniques, coupled with the fossil record, furnished detailed information of the absolute timing of lineage splits. Biogeographical hypothesis testing, based on Bayesian inference, parsimony and maximum likelihood methods, estimated the probability of temporal congruence and directionality of lineage connections (Crisp *et al.*, 2011).

Biogeographical congruence in Antirrhineae

Sampling strategy and DNA sequencing

In the Antirrhineae and most angiosperms, plastid DNA is usually inherited by ovules (Corriveau & Coleman, 1988), and is therefore preferred when reconstructing seed colonization (see Fernández-Mazuecos & Vargas, 2011). A total of 60 accessions of plastid (*ndh*F) sequences were included (see Table S1 in Appendix S1 of Supporting Information), representing 25 of the 27 genera of the tribe Antirrhineae following Sutton's (1988) taxonomic treatment plus two additional genera that have been recognized since (*Pseudomisopates* and *Nanorrhinum*; Güemes, 1997; Ghebrehiwet, 2001). Two New World genera (*Epixiphium*, *Holmgrenanthe*) were excluded due to a lack of material; their placement within the *Cymbalaria* clade in a previous internal transcribed spacer (ITS) phylogeny and the congruence between major clades of the ITS and *ndh*F analyses (see below) indicate that excluding these two genera does not significantly affect our analyses (see Fernández-Mazuecos *et al.*, 2013). As a lack of monophyly has rarely been found in Antirrhineae genera (Vargas *et al.*, 2004; Fernández-Mazuecos *et al.*, 2013), only one accession was sampled per genus, although two accessions of the only amphi-Atlantic genus of Antirrhineae, Old World *Linaria* and New World *Linaria* (formerly *Nuttallanthus*) were included (see Fernández-Mazuecos *et al.*, 2013). As a result, 19 new sequences of Antirrhineae were generated for this study and eight were retrieved from GenBank. Outgroup taxa were chosen to represent both closely related and more distant families for which reliable fossils are available, and consisted of 33 sequences in GenBank from the order Lamiales, including the families Plantaginaceae, Bignoniaceae, Acanthaceae, Orobanchaceae, Lamiaceae, Verbenaceae, Pedaliaceae, Scrophulariaceae, Gesneriaceae, Calceolariaceae, Oleaceae and Plocospermataceae. See Appendix S2 for a full description of DNA sequencing.

Phylogenetic analysis

The sequences were aligned using MAFFT 6 (Katoh & Toh, 2008) with minor adjustments made by visual inspection. Bayesian inference (BI), maximum-likelihood (ML) and maximum-parsimony (MP) analyses were conducted. To determine the optimal model of sequence evolution that best fitted the sequence data (GTR+I+G), the Akaike information criterion (AIC) was implemented using jModelTest 0.1.1 (Posada, 2008). See Appendix S2 for a full description of phylogenetic analyses.

Bayesian dating

To estimate divergence times among Lamiales lineages, including the Antirrhineae genera, we analysed the *ndh*F matrix through a relaxed molecular clock approach implemented in BEAST 1.6.1 (Drummond & Rambaut, 2007). No reliable fossils of the Antirrhineae appropriate for molecular calibration have yet been discovered (Martínez-Millán, 2010). We therefore employed a previous molecular estimate and five Lamiales fossils as constraints. All fossils have been considered reliable and proposed as calibration points for molecular dating in previous studies (Besnard *et al.*, 2009; Martínez-Millán, 2010; Thiv *et al.*, 2010). See Table 1 and Appendix S2 for further details.

Ancestral-area reconstructions

Biogeographical reconstructions were conducted delimiting just two areas (Old World/New World), and ancestors were allowed to be present in both. First, we analysed the BEAST output trees using statistical dispersal–vicariance analysis (S-DIVA), an approach to parsimony-based dispersal–vicariance analysis (DIVA; Ronquist, 1997) that accounts for phylogenetic uncertainty. S-DIVA analyses were conducted following the methods of Harris & Xiang (2009) implemented in the program RASP 1.1 (Yu *et al.*, 2011). All outgroup taxa except *Lafuentea* (the sister group to the Antirrhineae) were pruned from the trees. We used 1000 randomly sampled post-burn-in trees from the BEAST run, and the maximum clade credibility tree as the reference tree for summarizing S-DIVA results.

We also performed dispersal–extinction–cladogenesis analysis (DEC; Ree & Smith, 2008), a parametric likelihood-based approach, to reconstruct ancestral distributions. DEC

Table 1 Calibration points, associated fossils and minimum age constraints employed in the relaxed-clock analysis of *ndh*F sequences of Lamiales (including Antirrhineae genera). Nodes are named as in Fig. S1 in Appendix S1.

Fossil (Family/tribe)	Node	Time interval	Minimum age (Ma)	Reference
Fraxinus wilcoxiana (Oleaceae)	A	middle Eocene	37.2	Call & Dilcher (1992)
Catalpa rugosa (Bignoniaceae)	B	early–middle Oligocene	28.4	Reid & Chandler (1926)
Ajuginucula smithii (Lamiaceae)	C	early–middle Oligocene	28.4	Reid & Chandler (1926)
Gratiola tertiaria (Plantaginaceae/Gratioleae)	D	Miocene	5.3	Łańcucka-Środoniowa (1977)
Plantaginacearumpollis (Plantaginaceae s.str.)	E	middle Miocene	11.6	Nagy (1963)

analysis estimates the most likely geographical distribution of the two daughter lineages following a speciation event. Thus, whereas S-DIVA reports the ancestral range prior to the speciation event, DEC reports how the ancestral range is divided between the two descendants immediately after speciation. DEC analysis was implemented using Lagrange 2.0.1 (Ree & Smith, 2008). We set symmetric dispersal between areas, and constant dispersal rates through time, given that the sea barrier between the Old World and the New World formed in the Eocene (Axelrod, 1975) and has remained for the entire duration of diversification in Antirrhineae indicated by the dating analysis (see below).

Temporal congruence

To statistically evaluate the temporal congruence of the amphi-Atlantic disjunctions found across the Antirrhineae, we compared divergence-time estimates for the four Old World/New World lineages across the combined posterior distribution of 72,000 trees taken from the beast analysis. Divergence times between Old World and New World lineages of Antirrhineae constitute good estimates of colonization times if we assume that disjunctions are the result of long-distance dispersal (see below). Divergence-time estimates for the four Old World/New World lineages were first compared by inspecting the marginal density distributions of time to the most recent common ancestors (TMRCAs) in Tracer 1.4 (Rambaut & Drummond, 2007). We then obtained the posterior probability (PP) of occurrence of each divergence within the boundaries of each geological epoch, as defined by the *International Stratigraphic Chart 2009* (available at http://www.stratigraphy.org/). This probability was calculated as the proportion of trees from the posterior distribution where a particular Old World/New World lineage divergence fell into the bounds of a certain geological epoch. Finally, we calculated the PP of temporal congruence of two or more split events as the proportion of trees in which two or more Old World/New World lineage divergences occurred within the bounds of the same geological epoch. In order to provide statistical support for the BCH, PPs above 0.50 were classified into the categories 'high' ($0.90 \leq PP \leq 1.00$), 'medium' ($0.75 \leq PP < 0.90$) and 'low' ($0.50 \leq PP < 0.75$).

In order to test whether the four colonization events of the New World all occurred synchronously within the Miocene (as suggested by previous analyses) with a higher probability than expected by chance, we generated a null distribution of the timings of the four divergence events. To this end, we produced a distribution of trees with the same topology and the same root age as obtained in the maximum clade credibility (MCC) tree of the Bayesian dating analysis, but allowing the internal branch lengths to vary according to a birth–death model. We fixed the tree topology in beast by setting the MCC tree as the starting tree and unselecting all Markov chain Monte Carlo (MCMC) operators that act on the tree model. Analyses were repeated for two different fixed root ages – 69.3 and 105.2 Ma – representing the upper and lower 95% highest posterior density limits of the root age obtained in the MCC tree. For each analysis, we conducted five independent runs of 20 million generations, thus obtaining a total 10,000 trees. We then calculated the percentage of trees that had all four colonization events occurring between 23.03 and 5.33 Ma (Miocene).

Testing the Madrean–Tethyan hypothesis across angiosperm groups

In addition to the Antirrhineae, five of the 28 angiosperm groups considered to support the Madrean–Tethyan hypothesis (Raven & Axelrod, 1978; Kadereit & Baldwin, 2012) were analysed: Arbutoideae (Hileman *et al.*, 2001), *Cercis* (Davis *et al.*, 2002), Cistaceae (Guzmán & Vargas, 2009), Geraniaceae (Fiz-Palacios *et al.*, 2010) and *Platanus* subgen. *Platanus* (Feng *et al.*, 2005). These plant groups were chosen because they fit the assumptions for testing the BCH, i.e. molecular phylogenies based on significant taxonomic sampling and appropriate fossil-based calibration points or molecular substitution rates for phylogenetic tree ultrametrization are available. The five original DNA sequence datasets from published studies were obtained directly from the authors. For each analysis, we used the strategy used for the Antirrhineae (see above, Table S2 in Appendix S1, and Appendix S2).

RESULTS

Biogeographical congruence in Antirrhineae

Phylogenetic analyses

The *ndh*F matrix contained 2086 bp and had 1047 variable sites, of which 686 were parsimony-informative. The phylogenetic analyses revealed that the Antirrhineae constitute a monophyletic group (see Fig. S1 in Appendix S1). All three

phylogenetic analyses (BI, ML and MP) recognized three well-supported lineages formed by Old World/New World sister groups: the *Cymbalaria* lineage (PP = 1; ML-BS = 99.4%; MP-BS = 97%), the *Linaria* lineage (PP = 1; ML-BS = 100%; MP-BS = 100%) and the *Galvezia* lineage (PP = 1; ML-BS = 100%; MP-BS = 100%) (Fig. S1 in Appendix S1; see also Fig. 1). One additional Old World/New World sister clade (the *Sairocarpus* lineage) had lower support (PP = 0.66; ML-BS = 63.2%; MP-BS = 63%) (Fig. S1 in Appendix S1).

Bayesian dating

Standard deviation of the uncorrelated lognormal relaxed clock (0.748) and coefficient of variation (0.783) for rate heterogeneity within our *ndh*F dataset indicated the presence of rate heterogeneity among lineages. Analysis with Tracer 1.4 (Rambaut & Drummond, 2007) confirmed that sample sizes were adequate, with effective sample size (ESS) values above 300 and plots showing equilibrium after discarding the burn-in. The topology of the MCC tree (see Fig. S1a in Appendix S1) was congruent with those of the ML and MP analyses (Fig. S1b–c in Appendix S1). Diversification of the major lineages of Antirrhineae may have occurred in the last 35 Myr, from the Oligocene onwards. Mean ages of the four MRCAs of Old World/New World lineages all fell within the Miocene (Fig. 1), making the possibility of vicariance as an explanation for these disjunctions unlikely.

Ancestral area reconstructions

Biogeographical analyses supported four migrations from the Old World to the New World. In the ancestral range reconstruction using S-DIVA, Old World-to-New World

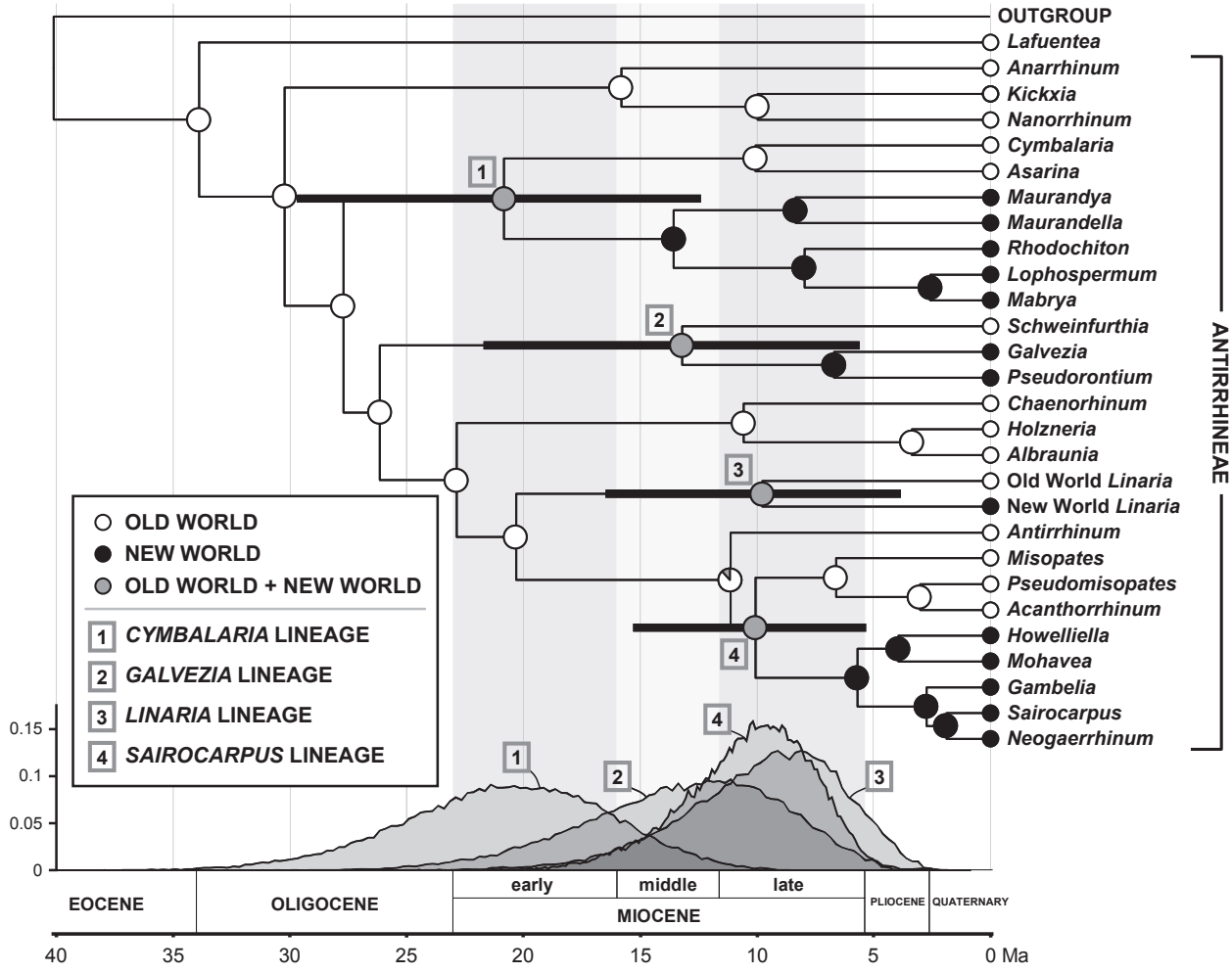

Figure 1 Molecular dating analysis and biogeographical reconstruction of Antirrhineae based on *ndh*F sequences. The maximum clade credibility tree produced by relaxed molecular clock analysis is shown. Outgroup lineages (except *Lafuentea*) have been pruned for clarity. Terminal circles indicate current distributions, and cicles or pie charts at nodes represent marginal probabilities for ancestral areas as inferred by S-DIVA analysis. Node bars in black represent the 95% highest posterior density intervals for time to the most recent common ancestors (TMRCAs) of four Old World/New World lineages (1–4). The marginal densities of TMRCAs of these four lineages are represented along the time-scale.

colonization events were strongly supported for the four lineages: *Cymbalaria* (node 1), *Galvezia* (node 2), *Linaria* (node 3) and *Sairocarpus* (node 4) (see Fig. 1; see also Fig. S2b in Appendix S1). DEC results were also congruent with an Old World-to-New World pattern for the four dispersal events (see Fig. S2a).

Temporal congruence

We compared the posterior distributions of the four TMRCAs (Fig. 1) to assess the temporal congruence of the four disjunction events. High PPs for the Miocene were obtained in the *Sairocarpus* (0.99), *Galvezia* (0.97) and *Linaria* (0.93) lineages (Table 2). For the *Cymbalaria* lineage, the most likely epoch was the Miocene (0.70), followed by the Oligocene (0.29). Within the Miocene, the middle–late subdivision received the highest PP for the *Sairocarpus* (0.96), *Linaria* (0.88) and *Galvezia* (0.74) lineages, whereas the early–middle Miocene received the highest PP (0.69) for the *Cymbalaria* lineage (Table 2). Furthermore, Table 3 shows the probabilities of temporal congruence for Antirrhineae, i.e. the PP that two, three or four TMRCAs occurred within the bounds of the same geological epoch. The Miocene was the only epoch that yielded significant probabilities, including a significant probability (PP = 0.62) of temporal congruence for the four lineages taken together. Interestingly, a medium to high probability value (PP = 0.89) was retrieved when considering only three lineages (*Galvezia*, *Linaria* and *Sairocarpus*). When subdividing the Miocene into three periods in order to narrow down a hypothetical migration period, the middle–late subdivision received the highest probability (PP = 0.63) for temporal congruence of these three lineages (Table 3). Therefore, we interpreted that optimal conditions for Old World/New World divergences within the Antirrhineae were concentrated in a period of about 10 million years (15.97–5.33 Ma).

The proportion of trees with a Miocene colonization to America by the four Antirrhineae lineages (62%; Table 3) was higher than that expected by chance. Indeed, we found that only 12.9% and 20% of trees of the null distributions (fixed root ages of 105.2 and 69.3 Ma, respectively) presented the four colonization events within the Miocene. These results provide further support for the hypothesis of a synchronous colonization of the American continent in the Miocene.

Testing the Madrean–Tethyan hypothesis across angiosperm groups

Our phylogenetic analyses suggested a higher number of Old World/New World lineage divergences than previously considered for these taxonomic groups (Raven & Axelrod, 1978). Our analyses detected 12 angiosperm lineages that include New World and Old World sister sublineages: four in Antirrhineae, one in Arbutoideae, two in *Cercis*, two in Cistaceae, two in Geraniaceae, and one in *Platanus* subgen. *Platanus* (Fig. 2a). Only 10 lineages could, however, be considered Madrean–Tethyan, because two were found to be very recent (Pliocene–Pleistocene): the eastern American species *Cercis canadensis* is closely related to the Mediterranean *C. siliquastrum*, and the New World species *Erodium texanum* is closely related to Old World *Erodium* lineages (Fig. 2a).

Biogeographical reconstructions for the six datasets indicated an Old World to New World directionality for the Antirrhineae, the two Cistaceae lineages and the basal disjunction of *Cercis* using S-DIVA and DEC analyses (Fig. 2a & Fig. S2). In contrast, a New World to Old World connection was supported for several other lineages (Arbutoideae, *Platanus*; Fig. 2a), and the directionality was equivocal for the *Erodium–California* (Geraniaceae) split. In the DEC analyses (see Fig. S2), an Old World to New World dispersal was obtained for Arbutoideae, Cistaceae (one to two events), *Platanus* and *Cercis*, as well as for the four Antirrhineae lineages. Differences between S-DIVA and DEC analyses are due to the different speciation modes modelled by the two methods. In S-DIVA, widespread ancestors are divided at speciation by allopatry, whereas DEC allows a widespread range to be inherited by one of the descendants. In fact, whereas dispersal events were always reconstructed along the branch preceding the MRCA of Old World/New World lineages in S-DIVA (Fig. 2a & Fig. S2), they were frequently reconstructed as an older event in the DEC analysis (see Fig. S2).

The posterior probabilities of the New/Old World splits for each of the five angiosperm groups (in addition to Antirrhineae) are shown in Table 2. In all cases, the divergence-time estimates for each of the Old World/New World divergences are congruent with the estimates obtained in the original publications. For the majority of the angiosperm groups, the Miocene was the epoch with the highest probability of occurrence of Old World/New World divergences (Fig. 2b). The mean probability of the Old World/New World disjunctions for the two datasets, i.e. the six non-Antirrhineae and the four Antirrhineae lineages, was high (0.85 PP) for the Miocene and very low for all other geological epochs (Table 2).

DISCUSSION

Historical biogeography of the six angiosperm groups provided poor support for an ancient (Palaeogene) Madrean–Tethyan flora, which is based on a vicariance hypothesis. Our approach extended the analysis to ten lineages from the six angiosperm groups examined. Interestingly, high levels of biogeographical congruence were found across plant lineages. Nevertheless, most of the intercontinental colonizations postdated the formation of the vicariance barrier of the Eocene (Tiffney & Manchester, 2001).

Synchronous amphi-Atlantic divergences of four independent snapdragon lineages

Our dating analysis demonstrates that Old World/New World lineage divergences of snapdragons (Antirrhineae)

Table 2 The posterior probability of the Old World/New World split of angiosperm lineages within the bounds of the six geological epochs of the Tertiary and five subdivisions of the Miocene.

	Six geological epochs of the Tertiary[a]						Three subdivisions of the Miocene[b]			Two subdivisions of the Miocene[c]	
Lineages	Eocene	Oligocene	Miocene	Pliocene	Pleistocene	Holocene	early Miocene	middle Miocene	late Miocene	early–middle Miocene	middle–late Miocene
Antirrhineae											
1. *Cymbalaria*	0.003	0.293	0.703*	< 0.001	< 0.001	< 0.001	0.569*	0.124	0.010	0.693*	0.135
2. *Galvezia*	< 0.001	0.018	0.969***	0.013	< 0.001	< 0.001	0.229	0.364	0.376	0.593*	0.740*
3. *Linaria*	< 0.001	< 0.001	0.927***	0.072	0.001	< 0.001	0.048	0.220	0.659*	0.268	0.879**
4. *Sairocarpus*	< 0.001	0.001	0.991***	0.008	< 0.001	< 0.001	0.036	0.269	0.686*	0.305	0.955***
Other angiosperm groups											
Platanus orientalis + *Platanus racemosa*	0.037	0.241	0.707*	0.014	0.001	< 0.001	0.316	0.219	0.172	0.535*	0.392
Lechea	< 0.001	< 0.001	0.999***	0.001	< 0.001	< 0.001	0.007	0.312	0.680*	0.319	0.992***
Crocanthemum / Hudsonia	< 0.001	< 0.001	0.774**	0.225	< 0.001	< 0.001	0.000	< 0.001	0.774**	< 0.001	0.774**
Erodium / California	< 0.001	< 0.001	1.000***	< 0.001	< 0.001	< 0.001	1.000***	< 0.001	< 0.001	1.000***	< 0.001
Arbutoideae Med. vs. NA mer.	0.023	0.387	0.589*	< 0.001	< 0.001	< 0.001	0.584*	0.006	< 0.001	0.589*	0.006
Cercis	< 0.001	0.210	0.789**	0.001	< 0.001	< 0.001	0.301	0.272	0.216	0.573*	0.489
Mean	0.006	0.115	0.845**	0.033	< 0.001	< 0.001	0.309	0.179	0.357	0.488	0.536*

*0.50 ≤ PP < 0.75; **0.75 ≤ PP < 0.90; ***0.90 ≤ PP.

[a]Eocene (55.8–33.9 Ma); Oligocene (33.9–23.03 Ma); Miocene (23.03–5.33 Ma); Pliocene (5.33–2.59 Ma); Pleistocene (2.59–0.01 Ma).

[b]Early Miocene, 23.03–15.97 Ma; middle Miocene, 15.97–11.61 Ma; late Miocene, 11.61–5.33 Ma.

[c]Early–middle Miocene, 23.03–11.61 Ma; middle–late Miocene, 15.97–5.33 Ma.

Table 3 Posterior probabilities (PP) of temporal congruence of two or more Old/New World lineage divergences of Antirrhineae in the six geological epochs of the Tertiary and five subdivisions of the Miocene.

	Six geological epochs of the Tertiary[a]						Three subdivisions of the Miocene[b]			Two subdivisions of the Miocene[c]	
Lineages[d]	Eocene	Oligocene	Miocene	Pliocene	Pleistocene	Holocene	early Miocene	middle Miocene	late Miocene	early–middle Miocene	middle–late Miocene
1,2	< 0.0001	0.0112	0.6868*	< 0.0001	< 0.0001	< 0.0001	0.1154	0.0445	0.0047	0.3887	0.1115
1,3	< 0.0001	0.0002	0.6441*	< 0.0001	< 0.0001	< 0.0001	0.0201	0.0163	0.0076	0.1556	0.1175
1,4	< 0.0001	0.0006	0.6958*	< 0.0001	< 0.0001	< 0.0001	0.0146	0.0196	0.0091	0.1736	0.1302
2,3	< 0.0001	< 0.0001	0.8982**	0.0019	< 0.0001	< 0.0001	0.0153	0.0803	0.2598	0.1754	0.6512*
2,4	< 0.0001	0.0001	0.9605***	0.0001	< 0.0001	< 0.0001	0.0135	0.0981	0.2832	0.2036	0.7119*
3,4	< 0.0001	< 0.0001	0.9190***	0.0007	< 0.0001	< 0.0001	0.0054	0.0757	0.4755	0.1143	0.8418**
1,2,3	< 0.0001	< 0.0001	0.6293*	< 0.0001	< 0.0001	< 0.0001	0.0054	0.0061	0.0038	0.0982	0.0980
1,2,4	< 0.0001	0.0001	0.6792*	< 0.0001	< 0.0001	< 0.0001	0.0048	0.0072	0.0043	0.1124	0.1075
1,3,4	< 0.0001	< 0.0001	0.6372*	< 0.0001	< 0.0001	< 0.0001	0.0020	0.0045	0.0065	0.0578	0.1133
2,3,4	< 0.0001	< 0.0001	0.8904**	< 0.0001	< 0.0001	< 0.0001	0.0019	0.0264	0.2032	0.0786	0.6277*
1,2,3,4	< 0.0001	< 0.0001	0.6225*	< 0.0001	< 0.0001	< 0.0001	0.0006	0.0013	0.0035	0.0389	0.0944

*0.50 ≤ PP < 0.75; **0.75 ≤ PP < 0.90; ***0.90 ≤ PP.

[a]Eocene (55.8–33.9 Ma); Oligocene (33.9–23.03 Ma); Miocene (23.03–5.33 Ma); Pliocene (5.33–2.59 Ma); Pleistocene (2.59–0.01 Ma).

[b]Early Miocene, 23.03–15.97 Ma; middle Miocene, 15.97–11.61 Ma; late Miocene, 11.61–5.33 Ma.

[c]Early–middle Miocene, 23.03-11.61 Ma; middle–late Miocene, 15.97–5.33 Ma.

[d]1, *Cymbalaria* lineage; 2, *Galvezia* lineage; 3, *Linaria* lineage; 4, *Sairocarpus* lineage.

considerably post-dated the Eocene, and therefore were established after an extensive water barrier (the Atlantic Ocean) had been formed between America and Europe (Tiffney & Manchester, 2001). Therefore, a vicariance process involving four independent splits of snapdragons is not supported by our data. The Bering land bridge between Asia and western North America could have provided a connection for temperate plant lineages into the late Miocene (Hong, 1983). Irrespective of particular geographical routes, our biogeographical reconstruction strongly supported four Old World-to-New World colonization events, followed by allopatric differentiation during the Miocene (Fig. 1). Indeed, the geographical distribution of the sister genus *Lafuentea* and the basal-most lineage of Antirrhineae (the *Anarrhinum* lineage) suggests a primary centre of diversification in the Old World. To our knowledge, this is the first time that biogeographical analyses statistically support a synchronous process of disjunction within a single plant group that is consistent with previous predictions for the BCH (Vargas *et al.*, 2004; Wen & Ickert-Bond, 2009).

The biogeographical congruence hypothesis (BCH) revisited

The process of geographical distribution being shared by two or more lineages that originated during the same period of time is known as biogeographical congruence (Page, 1990; Cunningham & Collins, 1994; Donoghue & Moore, 2003). Three conditions need to be met to reliably support the BCH: (1) similar distributions of taxa in two territories with comparable ecological conditions, e.g. disjunctions of Mediterranean/Californian lineages; (2) common ancestry of lineages, i.e. sister-group relationships of lineages currently forming part of comparable floras and faunas; and (3) synchrony of divergence times for sister lineages. As more angiosperm groups are found to show similar biogeographical patterns, i.e. multiple lineages exhibit the same disjunction that arose in the same period, greater support is given to biogeographical connectivity (Donoghue *et al.*, 2001; Xiang & Soltis, 2001; Wen & Ickert-Bond, 2009).

Our results do not support the vicariance hypothesis for the Madrean–Tethyan flora, but instead support a process in which multiple colonizations occurred following the establishment of a putative barrier (the Atlantic Ocean) (Crisp *et al.*, 2011). Testing the BCH is also dependent on the length of each geological period. For instance, temporal congruence of the snapdragon lineage divergences is high for the Miocene as a whole, but medium or low when dividing the Miocene into two or three subdivisions, respectively (Table 3). The likelihood of higher or lower biogeographical congruence is therefore closely related to the number of lineages involved and the window of opportunity offered by the duration of each geological period.

Biogeographical congruence also has ecological significance, because it suggests that evolution occurred under similar habitat conditions in two disjunct areas. We found four Antirrhineae disjunctions that fitted into the BCH for the Miocene, and also discovered two lineage splits in the Cistaceae in this geological epoch. In addition, the highest probability estimates for New World/Old World connections in the Geraniaceae and *Cercis* (one lineage each) were also discovered in the Miocene (Fig. 2b). The Miocene is thought to have been a period of expansion for temperate plant groups through northern corridors with similar habitat

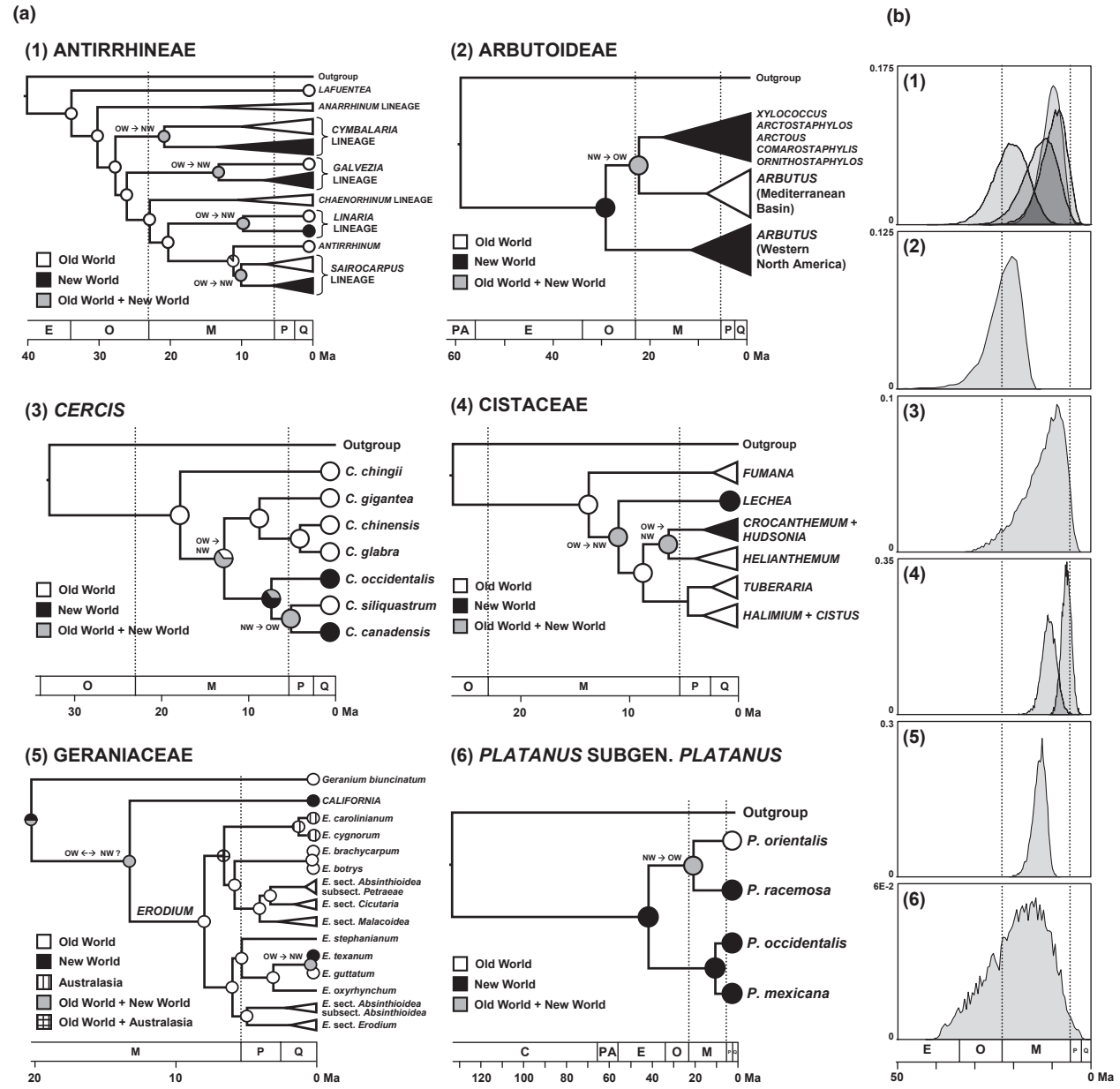

Figure 2 Summarized chronograms and biogeographical reconstructions of six analysed lineages of the Madrean–Tethyan flora. (a) Maximum clade credibility trees produced by relaxed molecular-clock analyses are shown. Mean ages of nodes are represented. Pie charts at nodes indicate marginal probabilities for ancestral areas as inferred by S-DIVA analysis. Shading and hatching of clade triangles represent the ancestral area (marginal probability = 1) of the clade. (b) Marginal densities of TMRCAs of Old World/New World clades belonging to the six analysed lineages of the Madrean–Tethyan flora. Two graphs (1, 4) display four and two disjunction nodes in the same plant group. Dotted lines mark the boundaries of the Miocene.

conditions that favoured intercontinental colonization (Grímsson *et al.*, 2007). In contrast, older biogeographical events pre-dating the opening of the oceanic gap between America and Eurasia cannot be ruled out for Arbutoideae (Hileman *et al.*, 2001) and *Platanus* (Feng *et al.*, 2005). Irrespective of specific support for vicariance or long-distance dispersal between America and Eurasia, the question remains as to whether shared ecological conditions in the same (biogeographical congruence) or different (biogeographical pseudocongruence) geological periods have been predominant in shaping the floras of California and the Mediterranean (Edwards *et al.*, 2007).

Legacy of Tertiary relict floras

Californian and Mediterranean floras appear to have included many closely related plant lineages since the Miocene, well before the onset of Mediterranean climates (Wen & Ickert-Bond, 2009; Kadereit & Baldwin, 2012). More recent connections between populations within species

(*Plantago ovata*, Meyers & Liston, 2008; *Oligomeris linifolia*, Martín-Bravo *et al.*, 2009), and between closely related species (*Erodium* spp., Fiz-Palacios *et al.*, 2010), also provide examples of more recent (Pleistocene) dispersal between Mediterranean floristic regions. These results lead us to conclude that taxonomic treatments constitute a good starting hypothesis regarding Pleistocene biogeographical divergence of lineages at low (intraspecific) taxonomic levels, while supraspecific levels better reflect lineage differentiation during earlier geological epochs of the Tertiary. Indeed, Raven & Axelrod (1978) proposed a Madrean–Tethyan flora primarily based on supraspecific taxa.

A continuous process of plant exchange between other temperate floras of North America and Asia has already been documented (Donoghue *et al.*, 2001; Xiang & Soltis, 2001). A review of 33 dated phylogenies of temperate vascular plants upheld the view that eastern Asia and eastern North America were connected by migrations at different times, but mostly in the Miocene (Donoghue & Smith, 2004). The same is true for Mediterranean/Californian elements, as recently reviewed for 25 angiosperm groups (Kadereit & Baldwin, 2012). Nevertheless, the high number of Mediterranean/Californian disjunctions (10 of the 12 angiosperm disjunctions tested in this paper) that shared a MRCA in the Miocene (Fig. 2), an epoch earlier than the establishment of both Mediterranean climates, is intriguing. The Mediterranean climates are thought to have become established in the Miocene–Pliocene (7–4 Ma; Millar, 2012) in California, and in the Pliocene (2.8 Ma; Suc, 1984) in the Mediterranean basin, when the Miocene floristic connections were already in place (Fig. 2b). Many Mediterranean plants adapted to summer drought are considered to be derived within lineages with earlier adaptations to drought (Millar, 2012). For instance, a drought-adapted Miocene (17–7 Ma) ancestor of Mediterranean–western American lineages has been proposed for *Lonicera* (a genus not included as Madrean–Tethyan in Raven & Axelrod, 1978), potentially conferring pre-adaptation to Mediterranean habitats (Smith & Donoghue, 2010). In conclusion, different degrees of similarity for both the Mediterranean and Californian floras can be estimated in light of floristic merging of ancient (Madrean–Tethyan), old (Miocene) and recent (Mediterranean climates) lineages.

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REFERENCES

Axelrod, D.I. (1975) Evolution and biogeography of Madrean-Tethyan sclerophyll vegetation. *Annals of the Missouri Botanical Garden*, **62**, 280–334.

Besnard, G., Rubio de Casas, R., Christin, P.-A. & Vargas, P. (2009) Phylogenetics of *Olea* (Oleaceae) based on plastid and nuclear ribosomal DNA sequences: Tertiary climatic shifts and lineage differentiation times. *Annals of Botany*, **104**, 143–160.

Call, V.B. & Dilcher, D.L. (1992) Investigations of angiosperms from the Eocene of southeastern North America: samaras of *Fraxinus wilcoxiana* Berry. *Review of Palaeobotany and Palynology*, **74**, 249–266.

Corriveau, J.L. & Coleman, A.W. (1988) Rapid screening method to detect potential biparental inheritance of plastid DNA and results for over 200 angiosperm species. *American Journal of Botany*, **75**, 1443–1458.

Crisp, M.D., Trewick, S.A. & Cook, L.G. (2011) Hypothesis testing in biogeography. *Trends in Ecology and Evolution*, **26**, 66–72.

Cunningham, C.W. & Collins, T.M. (1994) Developing model systems for molecular biogeography: vicariance and interchange in marine invertebrates. *Molecular ecology and evolution: approaches and applications* (ed. by B. Schierwater, B. Streit, G.P. Wagner and R. DeSalle), pp. 405–433. Birkhauser Verlag, Basel, Switzerland.

Davis, C.C., Fritsch, P.W., Li, J. & Donoghue, M.J. (2002) Phylogeny and biogeography of *Cercis* (Fabaceae): evidence from nuclear ribosomal ITS and chloroplast *ndhF* sequence data. *Systematic Botany*, **27**, 289–302.

Donoghue, M.J. & Moore, B.R. (2003) Toward an integrative historical biogeography. *Integrative and Comparative Biology*, **43**, 261–270.

Donoghue, M.J. & Smith, S.A. (2004) Patterns in the assembly of temperate forests around the Northern Hemisphere. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **359**, 1633–1644.

Donoghue, M.J., Bell, C.D. & Li, J. (2001) Phylogenetic patterns in Northern Hemisphere plant geography. *International Journal of Plant Sciences*, **162**, S41–S52.

Drummond, A.J. & Rambaut, A. (2007) BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology*, **7**, 214.

Edwards, E.J., Still, C.J. & Donoghue, M.J. (2007) The relevance of phylogeny to studies of global change. *Trends in Ecology and Evolution*, **22**, 243–249.

Feng, Y., Oh, S.-H. & Manos, P.S. (2005) Phylogeny and historical biogeography of the genus *Platanus* as inferred from nuclear and chloroplast DNA. *Systematic Botany*, **30**, 786–799.

Fernández-Mazuecos, M. & Vargas, P. (2011) Historical isolation *versus* recent long-distance connections between Europe and Africa in bifid toadflaxes (*Linaria* sect. *Versicolores*). *PLoS ONE*, **6**, e22234.

Fernández-Mazuecos, M., Blanco-Pastor, J.L. & Vargas, P. (2013) A phylogeny of toadflaxes (*Linaria* Mill.) based on nuclear internal transcribed spacer sequences: systematic and evolutionary consequences. *International Journal of Plant Sciences*, **174**, 234–249.

Fiz-Palacios, O., Vargas, P., Vila, R., Papadopulos, A.S.T. & Aldasoro, J.J. (2010) The uneven phylogeny and biogeography of *Erodium* (Geraniaceae): radiations in the Mediterranean and recent recurrent intercontinental colonization. *Annals of Botany*, **106**, 871–884.

Fritsch, P. (1996) Isozyme analysis of intercontinental disjuncts within *Styrax* (Styracaceae): implications for the Madrean–Tethyan hypothesis. *American Journal of Botany*, **83**, 342–355.

Ghebrehiwet, M. (2001) Taxonomy, phylogeny and biogeography of *Kickxia* and *Nanorrhinum* (Scrophulariaceae). *Nordic Journal of Botany*, **20**, 655–689.

Grímsson, F., Denk, T. & Símonarson, L.A. (2007) Middle Miocene floras of Iceland – the early colonization of an island? *Review of Palaeobotany and Palynology*, **144**, 181–219.

Güemes, J. (1997) *Pseudomisopates* (Scrophulariaceae), un nuevo género ibérico. *Anales del Jardín Botánico de Madrid*, **55**, 492–493.

Guzmán, B. & Vargas, P. (2009) Historical biogeography and character evolution of Cistaceae (Malvales) based on analysis of plastid *rbcL* and *trnL-trnF* sequences. *Organisms Diversity and Evolution*, **9**, 83–99.

Harris, A.J. & Xiang, Q.-Y. (2009) Estimating ancestral distributions of lineages with uncertain sister groups: a statistical approach to Dispersal–Vicariance Analysis and a case using *Aesculus* L. (Sapindaceae) including fossils. *Journal of Systematics and Evolution*, **47**, 349–368.

Hileman, L.C., Vasey, M.C. & Parker, V.T. (2001) Phylogeny and biogeography of the Arbutoideae (Ericaceae): implications for the Madrean-Tethyan hypothesis. *Systematic Botany*, **26**, 131–143.

Hong, D.-Y. (1983) The distribution of Scrophulariaceae in the Holarctic with species reference to the floristic relationships between eastern Asia and eastern North America. *Annals of the Missouri Botanical Garden*, **70**, 701–712.

Kadereit, J.W. & Baldwin, B.G. (2012) Western Eurasian–western North American disjunct plant taxa: the dry-adapted ends of formerly widespread north temperate mesic lineages – and examples of long-distance dispersal. *Taxon*, **61**, 3–17.

Katoh, K. & Toh, H. (2008) Recent developments in the MAFFT multiple sequence alignment program. *Briefings in Bioinformatics*, **9**, 286–298.

Łańcucka-Środoniowa, M. (1977) New herbs described from the Tertiary of Poland. *Acta Palaeobotanica*, **18**, 37–44.

Manos, P.S. & Donoghue, M.J. (2001) Progress in Northern Hemisphere biogeography: an introduction. *International Journal of Plant Sciences*, **162**, S1–S2.

Martín-Bravo, S., Vargas, P. & Luceño, M. (2009) Is *Oligomeris* (Resedaceae) indigenous to North America? Molecular evidence for a natural colonization from the Old World. *American Journal of Botany*, **96**, 507–518.

Martínez-Millán, M. (2010) Fossil record and age of the Asteridae. *Botanical Review*, **76**, 83–135.

Meyers, S.C. & Liston, A. (2008) The biogeography of *Plantago ovata* Forssk. (Plantaginaceae). *International Journal of Plant Sciences*, **169**, 954–962.

Millar, C.I. (2012) Geologic, climatic, and vegetation history of California. *The Jepson manual: vascular plants of California* (ed. by B.G. Baldwin, D.H. Goldman, D.J. Keil, R. Patterson and T.J. Rosatti), pp. 49–68. University of California Press, Berkeley, CA.

Morrone, J.J. & Crisci, J.V. (1995) Historical biogeography: introduction to methods. *Annual Review of Ecology and Systematics*, **26**, 373–401.

Nagy, E. (1963) Some new spore and pollen species from the Neogene of the Mecsek Mountains. *Acta Botanica Hungarica*, **9**, 387–404.

Page, R.D.M. (1990) Component analysis: a valiant failure? *Cladistics*, **6**, 119–136.

Posada, D. (2008) jModelTest: phylogenetic model averaging. *Molecular Biology and Evolution*, **25**, 1253–1256.

Rambaut, A. & Drummond, A.J. (2007) *Tracer v1.4*. University of Edinburgh, Edinburgh, UK. Available at: http://beast.bio.ed.ac.uk/Tracer.

Raven, P.H. (1972) Plant species disjunctions: a summary. *Annals of the Missouri Botanical Garden*, **59**, 234–246.

Raven, P.H. & Axelrod, D.I. (1978) Origin and relationships of the California flora. *University of California Publications in Botany*, **72**, 1–134.

Ree, R.H. & Smith, S.A. (2008) Maximum likelihood inference of geographic range evolution by dispersal, local extinction, and cladogenesis. *Systematic Biology*, **57**, 4–14.

Reid, E.M. & Chandler, M.E.J. (1926) *Catalogue of Cainozoic plants in the Department of Geology*. Vol. 1: *The Brembridge Flora*. British Museum (Natural History), London.

Ronquist, F. (1997) Dispersal–vicariance analysis: a new approach to the quantification of historical biogeography. *Systematic Biology*, **46**, 195–203.

Ronquist, F. & Sanmartín, I. (2011) Phylogenetic methods in biogeography. *Annual Review of Ecology, Evolution, and Systematics*, **42**, 441–464.

Smith, S.A. & Donoghue, M.J. (2010) Combining historical biogeography with niche modeling in the *Caprifolium* clade of *Lonicera* (Caprifoliaceae, Dipsacales). *Systematic Biology*, **59**, 322–341.

Suc, J.P. (1984) Origin and evolution of the Mediterranean vegetation and climate in Europe. *Nature*, **307**, 429–432.

Sutton, D.A. (1988) *A revision of the tribe Antirrhineae*. Oxford University Press, Oxford.

Takhtajan, A. (1986) *Floristic regions of the world*. University of California Press, Berkeley, CA.

Thiv, M., Thulin, M., Hjertson, M., Kropf, M. & Linder, H.P. (2010) Evidence for a vicariant origin of Macaronesian–Eritreo/Arabian disjunctions in *Campylanthus* Roth (Plantaginaceae). *Molecular Phylogenetics and Evolution*, **54**, 607–616.

Thorne, R.F. (1972) Major disjunctions in the geographic ranges of seed plants. *Quarterly Review of Biology*, **47**, 365–411.

Tiffney, B.H. & Manchester, S.R. (2001) The use of geological and paleontological evidence in evaluating plant phylogeographic hypotheses in the Northern Hemisphere Tertiary. *International Journal of Plant Sciences*, **162**, S3–S17.

Vargas, P., Roselló, J.A., Oyama, R. & Güemes, J. (2004) Molecular evidence for naturalness of genera in the tribe Antirrhineae (Scrophulariaceae) and three independent evolutionary lineages from the New World and the Old. *Plant Systematic and Evolution*, **249**, 151–172.

Wen, J. (1999) Evolution of eastern Asian and eastern North American disjunct distributions in flowering plants. *Annual Review of Ecology and Systematics*, **30**, 421–455.

Wen, J. & Ickert-Bond, S.M. (2009) Evolution of the Madrean–Tethyan disjunctions and the North and South American amphitropical disjunctions in plants. *Journal of Systematics and Evolution*, **47**, 331–348.

Xiang, Q.-Y. & Soltis, D.E. (2001) Dispersal-vicariance analyses of intercontinental disjuncts: historical biogeographical implications for angiosperms in the Northern Hemisphere. *International Journal of Plant Sciences*, **162**, S29–S39.

Yu, Y., Harris, A.J. & He, X. (2011) *RASP (Reconstruct Ancestral State in Phylogenies) 1.1*. Sichuan University, Chengdu. Available at: http://mnh.scu.edu.cn/soft/blog/RASP.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Supplementary Tables S1–S2 including plant material and angiosperm groups exhibiting a Madrean–Tethyan disjunction; and supplementary Figures S1–S2 including phylogenetic relationships of Lamiales and biogeographical reconstructions of the angiosperm groups.

Appendix S2 Supplementary methods on biogeographical congruence in Antirrhineae and testing the Madrean–Tethyan hypothesis across angiosperm groups.

BIOSKETCH

Our research group is broadly interested in evolution, biogeography and molecular systematics of primarily Mediterranean plant groups.

Author contributions: P.V. conceived the idea; E.C. and A.F. performed the lab work; L.M.V., J.L.B-P., I.L., B.G. and M.F.-M. conducted the analyses; and P.V. wrote the text. All authors contributed intellectually to analytical issues and data interpretation, and all commented on the text.

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Appendix 2

Fernández-Mazuecos et al. (2013), "Corolla morphology influences diversification rates in bifid toadflaxes (*Linaria* sect. *Versicolores*)"

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Corolla morphology influences diversification rates in bifid toadflaxes (*Linaria* sect. *Versicolores*)

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• *Background and Aims* The role of flower specialization in plant speciation and evolution remains controversial. In this study the evolution of flower traits restricting access to pollinators was analysed in the bifid toadflaxes (*Linaria* sect. *Versicolores*), a monophyletic group of ~30 species and subspecies with highly specialized corollas.
• *Methods* A time-calibrated phylogeny based on both nuclear and plastid DNA sequences was obtained using a coalescent-based method, and flower morphology was characterized by means of morphometric analyses. Directional trends in flower shape evolution and trait-dependent diversification rates were jointly analysed using recently developed methods, and morphological shifts were reconstructed along the phylogeny. Pollinator surveys were conducted for a representative sample of species.
• *Key Results* A restrictive character state (narrow corolla tube) was reconstructed in the most recent common ancestor of *Linaria* sect. *Versicolores*. After its early loss in the most species-rich clade, this character state has been convergently reacquired in multiple lineages of this clade in recent times, yet it seems to have exerted a negative influence on diversification rates. Comparative analyses and pollinator surveys suggest that the narrow- and broad-tubed flowers are evolutionary optima representing divergent strategies of pollen placement on nectar-feeding insects.
• *Conclusions* The results confirm that different forms of floral specialization can lead to dissimilar evolutionary success in terms of diversification. It is additionally suggested that opposing individual-level and species-level selection pressures may have driven the evolution of pollinator-restrictive traits in bifid toadflaxes.

Key words: Convergence, flower specialization, trait-dependent diversification, species selection, pollination, speciation, reversal, nectar spur, flower tube, toadflax, *Linaria* sect. *Versicolores*.

INTRODUCTION

Variation of flower morphological traits has long been considered to drive evolution and diversification of angiosperms (Darwin, 1862, 1877; Grant, 1949; Stebbins, 1970; Kay and Sargent, 2009; van der Niet and Johnson, 2012). Adaptation to different pollinator vectors (particularly animal pollinators) has been hypothesized to be a major force shaping flower morphology. This notion gave rise to the concept of pollination syndromes, i.e. sets of flower traits (shape, colour, nectar, scent) that have convergently evolved in distant plant lineages as an adaptation to particular pollinators (bees, birds, moths, etc.) (Faegri and van der Pijl, 1979; Fenster *et al.*, 2004). However, the concept of pollination syndromes, which relies on flower specialization, has been challenged in recent times (Waser *et al.*, 1996; Ollerton *et al.*, 2009). Specialization may still play a relevant role in plant speciation (Kay and Sargent, 2009), but syndrome shifts may not account for the majority of speciation events (e.g. Valente *et al.*, 2012). Therefore, rather than focusing on the evolution of syndromes, the investigation of particular traits, including their evolutionary trends, shifts and correlations, is probably more fruitful for understanding flower evolution in most plant lineages (Smith, 2010).

Traits that restrict the access of pollinators to flower rewards (nectar, pollen) are of exceptional interest because these physical barriers may have evolved as a specialization to particular pollinators. Variations in length and width of flower tubes and nectar spurs have been the subject of several studies (Herrera, 1990; Johnson and Steiner, 1997; Alexandersson and Johnson, 2002; Pérez *et al.*, 2004; Whittall and Hodges, 2007; Tripp and Manos, 2008). An extreme case of restriction of pollinator access is the personate corolla of snapdragons (*Antirrhinum*) and some relatives of the tribe Antirrhineae and the order Lamiales (Sutton, 1988; Endress, 1994; Kampny, 1995). These species display zygomorphic, gamopetalous, bilabiate corollas in which the lower lip is conspicuously arched upwards, constituting a palate. This structure closes access to pollen and nectar rewards, therefore making the mechanical opening of the corolla necessary for insect pollination. The personate corolla has long been considered as an adaptation to bee pollination (mellitophily), as insects other than bees would not be strong or heavy enough to open it (Hill, 1909; Müller, 1929; Sutton, 1988; Endress, 1992; Vargas *et al.*, 2010).

The relationships between changes in restrictive flower traits and diversification (speciation minus extinction) rates remain poorly understood. Nectar spurs have been hypothesized to

represent a key innovation that promotes species diversification by providing a mechanism of pre-zygotic reproductive isolation through differential pollinator visitation (Hodges and Arnold, 1995; Hodges, 1997; but see Hagen and Kadereit, 2003; Cacho *et al.*, 2010). On the other hand, it has been historically argued that ecological specialists usually evolve from generalists, and that specialization constitutes an evolutionary dead end, i.e. a derived state from which both reversal to a generalist state and shift to a different specialized state would be unlikely (Futuyma and Moreno, 1988). There are, however, many examples that contradict this idea (Gómez and Zamora, 2006). In particular, such a view has been challenged by phylogeny-based analyses of flower evolution (Armbruster and Baldwin, 1998; Tripp and Manos, 2008; Fleming *et al.*, 2009). Simultaneous estimations of rates of character change and state-dependent speciation/extinction rates across phylogenetic trees are crucial for a correct understanding of character evolution (Maddison, 2006; Goldberg and Igić, 2008). Recently developed methods (Maddison *et al.*, 2007; FitzJohn *et al.*, 2009; FitzJohn, 2010) enable such estimations and hold great promise for understanding flower evolution (Smith, 2010), yet they have rarely been applied in this context (but see Armbruster *et al.*, 2009; Smith *et al.*, 2010; Valente *et al.*, 2012).

Toadflaxes (*Linaria*) constitute the most species-rich (~150 species) genus of the snapdragon lineage (tribe Antirrhineae, Plantaginaceae) (Sutton, 1988). *Linaria* pollination has historically attracted the interest of botanists and evolutionary biologists (Sprengel, 1793; Darwin, 1876). Toadflaxes constitute a natural group (Vargas *et al.*, 2004; Fernández-Mazuecos *et al.*, 2013) and display a remarkably diverse array of flower traits whose evolution has not, however, been analysed in a phylogenetic framework to date. Several traits of *Linaria* flowers are potentially linked to pollinator specialization: they have a zygomorphic, bilabiate, usually personate corolla in which a spur of variable length is formed at the base of the lower lip (Fig. 1). The spur contains nectar dripping down from a nectary located at the base of the ovary (Valdés, 1970). The two pairs of anthers are placed at slightly different heights, with the stigma in the space between. While most species have well-developed palates that close access to the corolla throat, in some species belonging to sections *Versicolores*, *Macrocentrum* and *Lectoplectron* the palate is poorly developed, and access to the corolla throat is wide open (e.g. Fig. 1Q). This seems to be usually related to a narrowing of the corolla tube and a broadening of the lower lip (Viano, 1969; Sutton, 1980). Such morphology has been suggested to be related to pollination by long-tongued lepidopterans and dipterans (Hill, 1909; Sutton, 1980, 1988), while the typical personate corolla would be linked to bee pollination (Hill, 1909; Arnold, 1982; Sánchez-Lafuente, 2007; Carrió *et al.*, 2012).

Here we analysed the evolution of flower morphology in a clade of *Linaria* (sect. *Versicolores*) that displays remarkable flower diversity. In particular, we used phylogenetic and comparative methods to achieve the following objectives: (1) to get a deeper insight into the phylogenetic relationships within this lineage; (2) to evaluate intra- and inter-specific morphological variation of traits limiting pollinator access to nectar reward; (3) to analyse whether restrictive traits have exerted an effect on diversification rates; and (4) to reconstruct the evolutionary history of flower morphology and to investigate its potential links to pollinators.

MATERIALS AND METHODS

Study group and taxonomic treatment

Linaria sect. *Versicolores* (bifid toadflaxes) is an assemblage of ~25 species mainly distributed in the western Mediterranean region (Sutton, 1988) (see examples in Fig. 1). According to phylogenetic analyses based on both nuclear and plastid DNA markers (Fernández-Mazuecos and Vargas, 2011; Fernández-Mazuecos *et al.*, 2013) bifid toadflaxes constitute a monophyletic group within *Linaria*, formed by two well-supported sister groups: subsect. *Elegantes* (two species) and subsect. *Versicolores* (~23 species). All species are diploid ($2n = 12$) except for the tetraploid *L. hellenica* (reviewed by Sutton, 1988). Most species seem to be allogamous (Bruun, 1937; Valdés, 1970; Docherty, 1982; M. Fernández-Mazuecos, unpubl. res.). Section *Versicolores* is an ideal system for the evolutionary analysis of several flower traits that restrict the access of pollinators to nectar reward: spur length, tube width and palate development. Although morphological affinities among species have not been analysed in detail, some divergent traits have been described. At least the two species of subsect. *Elegantes* (*L. elegans* and *L. nigricans*) display a widely open corolla mouth and a narrow tube (Fig. 1P, Q), while species of subsect. *Versicolores* usually exhibit typical personate (closed) corollas with a wider tube (e.g. Fig. 1A, K). Some authors, however, have suggested that flowers of certain species of subsect. *Versicolores* (*L. incarnata*, *L. bipartita*; Fig. 1B, E) resemble those of subsect. *Elegantes* regarding their narrow tubes and broad lower lips (Viano, 1969; Sutton, 1988). In addition, sect. *Versicolores* exhibits a wide variation in spur length, including some of the shortest (*L. clementei*; Fig. 1C) and longest (*L. elegans*; Fig. 1P) spurs in the genus (Sutton, 1988; Sáez and Bernal, 2009). These traits seem to be associated with contrasting species diversities: only a few species display narrow tubes, and spurs as short as those of *L. clementei* seem to be rare. Nevertheless, inter- and intra-specific morphological variability has not been quantitatively assessed to date.

The potential effects of alpha taxonomy on diversification rate analyses have been pointed out by some authors (Marazzi and Sanderson, 2010; Valente *et al.*, 2010*b*). Indeed, correct species delimitation is crucial in obtaining accurate estimates of speciation and extinction rates. Therefore, we first conducted a review of the taxonomic literature (Viano, 1978*a*, *b*; Sutton, 1988; Dobignard, 1997; Fennane and Ibn Tattou, 1998; De Leonardis *et al.*, 1999; Tan and Iatrou, 2001; De Leonardis *et al.*, 2003; Gómiz, 2004; Hamdi *et al.*, 2009; Sáez and Bernal, 2009) and a survey of herbarium specimens mainly from two herbaria with a broad representation of *Linaria* sect. *Versicolores* specimens from Iberia (MA) and northern Africa (RNG) (see Supplementary Data Appendix S1). Although the first modern synthesis of the group is due to Viano (1978*a*, *b*), we generally adopted the more inclusive taxonomic treatment of Sutton (1988), except for some modifications detailed in Supplementary Data Appendix S2. In the end, we accepted 30 taxa (including species and subspecies; Table 1) that are morphologically and geographically cohesive.

Phylogenetic relationships and divergence times

Sampling strategy and DNA sequencing. We sampled a total of 45 specimens of *Linaria* sect. *Versicolores*, including

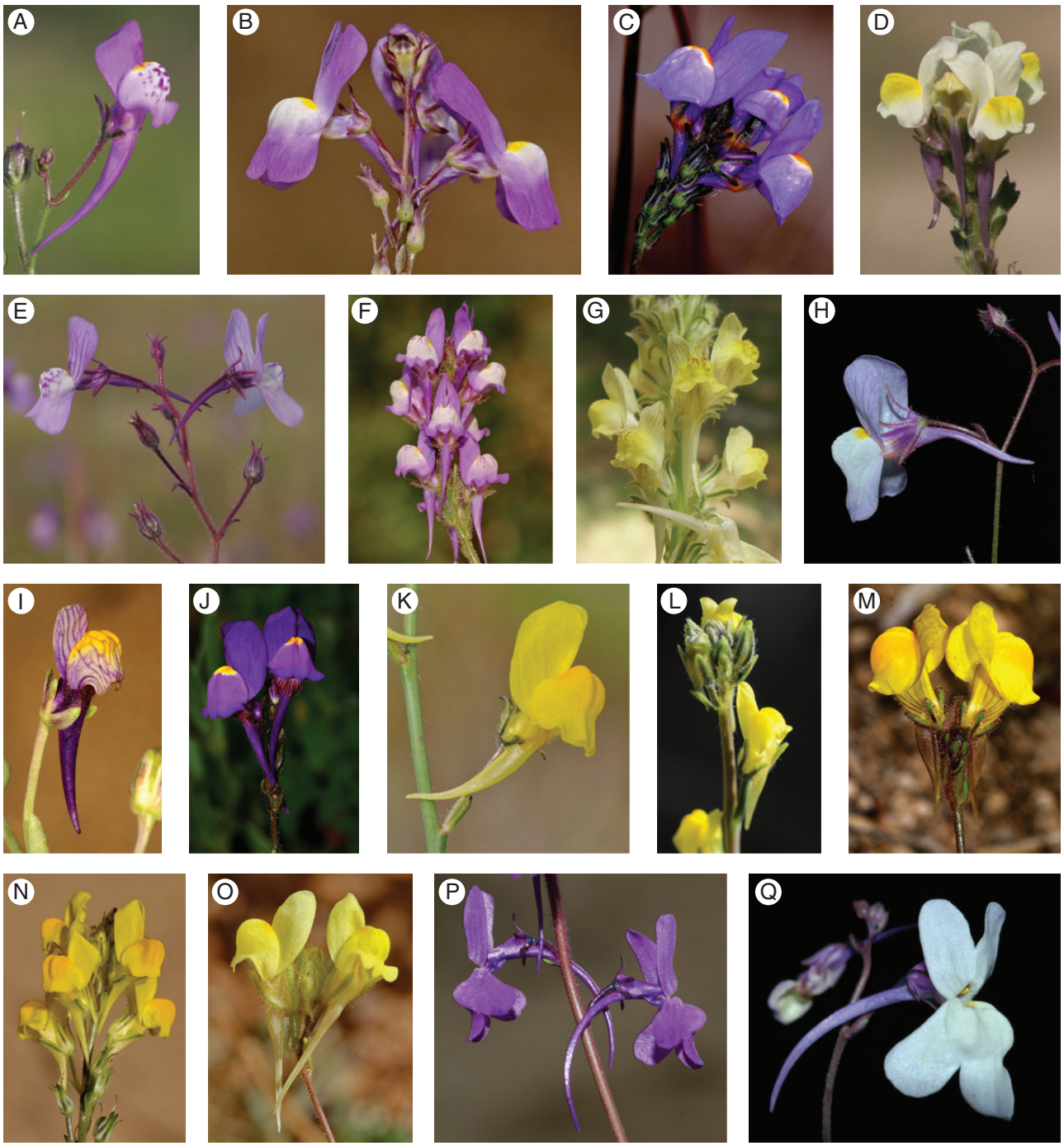


FIG. 1. Representatives of *Linaria* sect. *Versicolores*. Subsect. *Versicolores*: (A) *L. algarviana*; (B) *L. bipartita*; (C) *L. clementei*; (D) *L. gharbensis*; (E) *L. incarnata*; (F) *L. maroccana*; (G) *L. multicaulis* subsp. *heterophylla*; (H) *L. onubensis*; (I) *L. pedunculata*; (J) *L. salzmannii*; (K) *L. spartea*; (L) *L. tenuis*; (M) *L. viscosa* subsp. *spicata*; (N) *L. viscosa* subsp. *viscosa*; (O) *L. weilleri*. Subsect. *Elegantes*: (P) *L. elegans*; (Q) *L. nigricans*. Floral morphological types (see Fig. 4): Type I (broad tube, variable spur: A, D, F, G, I–O); Type II (broad tube, very short spur: C); Type III (narrow tube, variable spur: B, E, H, P, Q). Photographs by A. Fernández-Mazuecos (A, E), J. Quiles (B, F, K, N, O), J. Ramírez (C, I, J, M), J.L. Blanco-Pastor (D), E. Rico (G), P. Vargas (H, P, Q) and O. Fragman-Sapir (L).

representatives of 29 of the 30 recognized species and subspecies (one or two specimens per taxon; Table 1; Supplementary Data Table S1). To minimize the impact of recent hybridization, we selected unambiguously identified individuals, and some with intermediate traits or uncertain identification were discarded. We only failed to sample *L. dissita*, which is a poorly known northern African taxon (Sutton, 1988; Fennane and Ibn Tattou, 1998). We also sampled nine additional species representing six other sections of *Linaria*, one species of *Antirrhinum* and one of *Chaenorhinum* to be used as the outgroup based on previous phylogenetic evidence (Vargas *et al.*, 2004; Fernández-Mazuecos *et al.*, 2013). Plant material was collected in the

TABLE 1. *Taxonomic treatment followed in this paper, taxon distributions, individuals sampled for phylogenetic and morphometric analyses and flower morphological types*

Taxon	Distribution	No. individuals sampled for phylogenetic analyses	No. individuals sampled for spur and tube measures	No. individuals sampled for geometric morphometric analysis	Morphological type
***Linaria* Mill.**					
***Linaria* sect. *Versicolores* (Benth.) Wettst.**					
Subsect. *Versicolores*					
L. algarviana Chav.	SW Portugal (Algarve)	1	7	24	I
L. bipartita (Vent.) Willd.	W Morocco	2	46	—	III
L. bordiana Santa & Simonneau					
subsp. *bordiana*	Algeria	1	8	—	III
subsp. *kralikiana* (Maire) D. A. Sutton	NW Algeria	2	4	—	I
L. clementei Haens.	S Spain (Málaga)	2	22	21	II
L. dissita Pomel	NW Africa	—	6	—	I
L. gattefossei Maire & Weiller	C Morocco	1	6	—	I
L. gharbensis Batt. & Pit.	NW Africa, SW Spain	2	29	23	I
L. hellenica Turrill	S Greece	1	2	—	I
L. imzica Gómiz	S Morocco (Anti Atlas)	1	9	—	I
L. incarnata (Vent.) Spreng.	W Iberian Peninsula	2	36	48	III
L. mamorensis Mazuecos, Vigalondo & L. Sáez	NW Morocco	2	34	—	III
L. maroccana Hook.f.	Morocco (mainly High Atlas)	2	31	—	I
L. multicaulis (L.) Mill.					
subsp. *multicaulis*	Sicily, S Italy (Calabria)	1	4	—	I
subsp. *aurasiaca* (Pomel) D.A.Sutton	Tunisia, NE Algeria	1	3	—	I
subsp. *galioides* (Ball) D. A. Sutton	Morocco (High Atlas)	2	31	—	I
subsp. *heterophylla* (Desf.) D. A. Sutton	NW Africa	2	46	—	I
L. onubensis Pau	SW Spain (Huelva)	2	15	45	III
L. pedunculata (L.) Chaz.	S Iberian Peninsula, NW Africa, Balearic Islands	2	39	27	I
L. pinifolia (Poir.) Thell.	Tunisia, Algeria	1	5	—	I
L. pseudoviscosa Murb.	Tunisia	1	7	—	I
L. salzmannii Boiss.	S Spain (Málaga)	1	5	20	I
L. spartea (L.) Chaz.	Iberian Peninsula, S France	2	87	25	I
L. tenuis (Viv.) Spreng.	N Africa, Middle East	2	3	—	I
L. tingitana Boiss. & Reuter	NW Africa	1	12	—	I
L. viscosa (L.) Chaz.					
subsp. *viscosa*	S Iberian Peninsula	2	60	45	I
subsp. *spicata* (Kunze) D. A. Sutton	SE Iberian Peninsula	1	45	24	I
L. weilleri Emb. & Maire	S Morocco (Anti Atlas)	1	4	—	I
Subsect. *Elegantes* (Viano) D. A. Sutton					
L. elegans Cav.	NW Iberian Peninsula	2	58	24	III
L. nigricans Lange	SE Spain (Almería)	2	32	43	III

field and dried in silica gel or obtained from herbarium collections (RNG, MA, ATH, UPOS; Supplementary Data Table S1).

For phylogenetic analyses, we selected one nuclear (ITS) and three plastid (*rpl*32-*trn*L^{UAG}, *trn*K-*mat*K and *trn*S-*trn*G) DNA regions employed in our previous phylogenetic and phylogeographic analyses of the genus *Linaria* (Fernández-Mazuecos and Vargas, 2011; Blanco-Pastor *et al.*, 2012, 2013; Fernández-Mazuecos *et al.*, 2013; Fernández-Mazuecos and Vargas, 2013). One hundred and eighty-one sequences were taken from our previous studies, while the remaining 43 were newly generated. Procedures used for DNA extraction, amplification and sequencing of DNA regions followed Fernández-Mazuecos and Vargas (2011) and Fernández-Mazuecos *et al.* (2013). Sequences of each DNA region were separately aligned using MAFFT 6 (Katoh *et al.*, 2002) with default parameters, and further adjustments were made by visual inspection. The three ptDNA regions were concatenated in a single matrix after congruence was confirmed in preliminary phylogenetic analyses. All new

sequences have been deposited in the GenBank database (see Supplementary Data Table S1 for accession numbers).

Gene tree estimation and dating. Separate phylogenetic analyses were conducted on the ITS and ptDNA matrices using three methods: Bayesian inference (BI; implemented in MrBayes v3·1·2; Ronquist and Huelsenbeck, 2003); maximum likelihood (ML; implemented in RaxML; Stamatakis, 2006); and maximum parsimony (MP; implemented in TNT 1·1; Goloboff *et al.*, 2003) (see Supplementary Data Appendix S2 for details). Based on previous phylogenetic evidence (Vargas *et al.*, 2004), *Chaenorhinum* was used as the outgroup sequence in all analyses.

In order to obtain time-calibrated gene trees, separate ITS and ptDNA matrices including a single individual per species were analysed through the relaxed molecular clock approach implemented in BEAST 1·6·2 (Drummond *et al.*, 2006; Drummond and Rambaut, 2007). Following previous dating analyses of *Linaria* sect. *Versicolores* (Fernández-Mazuecos and Vargas, 2011, 2013), the root node (divergence between *Chaenorhinum* and *Linaria*) was calibrated using a normal distribution with mean 23 million years ago (Ma) and standard deviation 4 million years (Myr). This was based on a dating analysis of *ndh*F sequences of the tribe Antirrhineae (P. Vargas *et al.*, in prep.), which in turn incorporates a calibration of 74 Ma for the divergence time between Oleaceae and Antirrhineae (Bell *et al.*, 2010), and minimum stem-age constraints for Lamiales families and tribes based on five fossils (see Fernández-Mazuecos and Vargas, 2011 for details). Models of nucleotide substitution were selected for each DNA region under the Akaike information criterion (AIC) in jModelTest 0·1 (Posada, 2008). A birth–death process (Gernhard, 2008) was employed as tree prior. The substitution rate variation was modelled using an uncorrelated log-normal distribution. Based on previous estimates for herbaceous plants, uniform prior distributions were set for the substitution rates, with a range of 5×10^{-4} to 5×10^{-2} substitutions per site per Myr for ITS and 1×10^{-4} to 1×10^{-2} substitutions per site per Myr for ptDNA (see Blanco-Pastor *et al.*, 2012 for details). For each dataset, four Markov chain Monte Carlo (MCMC) analyses with 10 million generations each and a sample frequency of 1000 were run through the CIPRES Science Gateway (Miller *et al.*, 2010). Parameter analysis in Tracer 1·5 (Rambaut and Drummond, 2007) showed adequate chain length, with effective sample sizes above 1000. Chains were combined using LogCombiner 1·6·2 after discarding the first 10 % of sampled generations as burn-in. Trees were summarized in a maximum clade credibility (MCC) tree obtained in TreeAnotator 1·6·2 and visualized in FigTree 1·3·1.

Significant incongruence between loci prevented us from concatenating the ITS and ptDNA sequences for total-evidence phylogenetic and dating analyses (Kubatko and Degnan, 2007; Edwards, 2009). Instead, we implemented a species tree estimation analysis (see below).

Species tree estimation. Phylogenetic incongruence between loci is frequently found in plants, particularly in *Linaria*, due to incomplete lineage sorting and hybridization, among other causes (Blanco-Pastor *et al.*, 2012). While no standard method is currently available for the inference of phylogenetic relationships in the presence of these two processes, a number of coalescent-based methods have been recently proposed for the inference of species trees that account for incongruence between gene trees caused by incomplete lineage sorting (Liu, 2008; Heled and Drummond, 2010). Here we employed the ITS and ptDNA datasets including one or two individuals per taxon to estimate a species phylogeny of *Linaria* sect. *Versicolores* under the multispecies coalescent method *BEAST (Heled and Drummond, 2010), implemented in BEAST 1·6·2.

Haplotypic data are needed for coalescent-based analyses, which posed a challenge in the case of the multi-copy ITS region. Cloning of ITS copies was not considered due to the low quality of DNA extracts and PCR products obtained from herbarium material. Instead, in order to reconstruct haplotypes from the unphased ITS sequences, we employed the Bayesian statistical method PHASE 2·1 (Stephens *et al.*, 2001; Stephens and Donnelly, 2003), as implemented in DnaSP v5 (Librado and Rozas, 2009), with default parameters (recombination model MR0, 100 iterations, 100 burn-in iterations, thinning interval 1). A Bayesian phylogenetic analysis of the inferred haplotypes was conducted in MrBayes. Given that close (or unresolved) relationships between haplotypes of the same individual were recovered in all cases (Supplementary Data Fig. S1), the error introduced by potentially incorrect haplotype inference was considered negligible. Therefore, all ITS haplotypes inferred by PHASE were included in subsequent analyses following Blanco-Pastor *et al.* (2012).

Both datasets (ITS and ptDNA) were included as independent loci in the *BEAST analysis. The tree model and substitution model priors were set as indicated above for dating analyses. Based on results of separate dating analyses of ITS and ptDNA sequences (see above), the crown age of sect. *Versicolores* was calibrated using a normal prior with mean 6·07 Ma and standard deviation 1·85 Myr. Twenty MCMC analyses were run for 100 million generations each, with a sample frequency of 10 000. Analysis with Tracer 1·5 confirmed convergence and adequate sample sizes, with effective sample sizes above 250. Runs were combined using LogCombiner 1·6·2, after discarding the first 10 % of sampled generations as burn-in. Trees were summarized in an MCC tree obtained in TreeAnotator 1·6·2 and visualized in FigTree 1·3·1. In order to visualize the temporal dynamics of *Linaria* sect. *Versicolores* diversification, lineage-through-time (LTT) plots were generated in the R package *ape* (Paradis *et al.*, 2004) for the MCC species tree and a random sample of 1000 trees from the posterior distribution of the *BEAST analysis.

Analysis of corolla shape

Metric measures. In the personate corolla of *Linaria*, the main reward for pollinators (nectar) is located at the end of an abaxial spur of variable length (Fig. 1). Three main traits determine nectar accessibility to pollinators: spur length, tube width and palate development. Of these, spur length and tube width can be readily measured in herbarium specimens. In order to characterize the inter- and intra-specific variability of these traits in bifid toadflaxes, the two variables were scored for 696 herbarium specimens representing the 30 recognized species and subspecies of *Linaria* sect. *Versicolores* (Supplementary Data Appendix S1). Most specimens were provided by the MA, RNG and ATH herbaria. In addition, specimens from the MPU, RAB, FI, K, BM, LD and S herbaria were electronically surveyed through JSTOR Plant Science (Gallagher, 2010). A single, fully developed flower per specimen was measured.

Spur length was measured from the corolla–calyx insertion to the spur tip (Fig. 2A, C). Tube width was measured at the opening level (Fig. 2B, D). In addition, the same variables were measured for 377 living specimens collected from 18 Iberian populations of 12 representative species and subspecies sampled for geometric morphometric analyses (see below). All measurements were obtained from scaled digital photographs using ImageJ 1·44p (Abràmoff *et al.*, 2004).

Geometric morphometrics. Geometric morphometrics provides a powerful tool to assess intra- and inter-specific variation in flower morphology (Shipunov and Bateman, 2005; Gómez *et al.*, 2006; Abdelaziz *et al.*, 2011). We used a landmark-based geometric morphometric analysis to describe corolla shape, including palate development, in 18 populations belonging to 12 species and subspecies of *Linaria* sect. *Versicolores* from the Iberian Peninsula (Supplementary Data Table S2). These species were considered to represent the full range of corolla shapes of sect. *Versicolores* based on our metric measures of herbarium specimens of all 30 species and subspecies (see Results). A total of 369 living specimens (7–25 per population; Supplementary Data Table S2) were sampled, and digital photographs were taken of one completely developed flower per individual in lateral view and planar position. Nine landmarks (Fig. 2A, C) were defined at points of evident homology across species (Zelditch *et al.*, 2004). In addition, one pseudolandmark was defined at the mid-point of the spur. Landmarks were captured using tpsDig 2·16 (Rohlf, 2010). The two-dimensional coordinates of the landmarks were determined for each individual, and the generalized orthogonal least-squares Procrustes average configuration of landmarks was calculated using the generalized Procrustes analysis (GPA) superimposition method (Rohlf and Slice, 1990; Slice, 2001). Corolla shape differences among species were assessed using a canonical variate analysis, a multivariate analysis that optimizes between-group differences relative to within-group variation (Albrecht, 1980; Klingenberg and Monteiro, 2005). It generates several canonical variate axes and computes between-group Procrustes distances in the canonical variate space. The analysis was performed with the software MorphoJ (Klingenberg, 2011). Values of canonical variates 1 and 2 for all individuals were plotted. The statistical significance of the between-groups Procrustes distances was determined by randomization tests using 10 000 permutations.

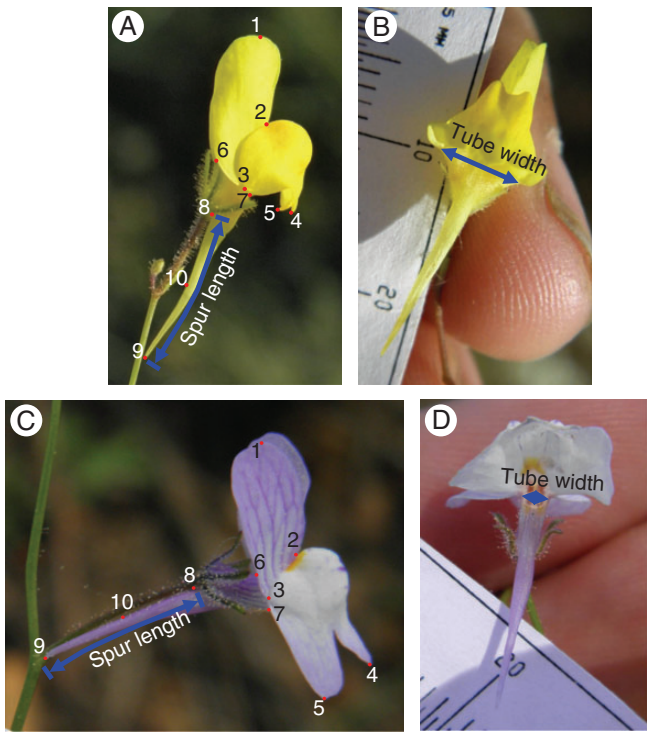


FIG. 2. Metric measures (spur length and tube width) and landmarks (1–10) employed in morphometric analyses, shown in two representative taxa of morphological Types I (*L. spartea*; A, B) and III (*L. onubensis*; C, D). Photos show lateral (A, C) and ventral (B, D) views. Notice the broad tube in B and the narrow tube in D.

Evolution of flower morphology

Effect of flower morphology on diversification rate. Morphometric analyses allowed the identification of three major floral morphological types (see Results). We estimated the effect of flower morphology on *Linaria* sect. *Versicolores* diversification using the binary-state speciation and extinction (BiSSE) model (Maddison *et al.*, 2007; FitzJohn *et al.*, 2009) implemented in the R package *diversitree* v.0·7–2 (FitzJohn, 2012). We defined two character states: (0) wide-tubed flower (Types I and II, see below); and (1) narrow-tubed flower (Type III) (Table 1). The fact that Type II was found in a single species (*L. clementei*) prevented us from using the multiple state speciation and extinction (MuSSE) method, which is an extension of BiSSE for more than two character states (FitzJohn, 2012). Instead, Type II was grouped with Type I based on their morphometric similarity (see below). All recognized taxa (species and subspecies) were included as independent entities in this analysis, based on the fact that subspecies belonging to the same species were usually not closely related in phylogenetic analyses (see below). The MCC species tree from the *BEAST analysis [with nodes with posterior probability (PP) <0·5 collapsed] and 10 additional species trees randomly chosen from the Bayesian posterior distribution were analysed. For each tree, we compared a model with state-dependent speciation and extinction and asymmetrical transition rates against nested models with speciation, extinction and transition rate parameters constrained to be equal for both states. We calculated ML parameter values of the unconstrained model (full BiSSE model, six parameters) versus the constrained models (five parameters), and the significance of model differences was assessed by performing likelihood ratio tests. Parameter values of the full BiSSE model were additionally explored for the MCC tree and the ten additional trees in a two-step process using ML values as a prior for an MCMC sampling of parameters, a Bayesian approach (MCMC-BiSSE) that provides a measure of parameter uncertainty (FitzJohn *et al.*, 2009). The trees were analysed with 10 000 steps per tree (chain) and a prior for each parameter exponentially distributed (prioritizing small rates of change, in the absence of evidence to the contrary). After discarding the first 2000 steps of each chain as burn-in, parameter values for each tree were summarized and plotted.

Reconstruction of flower morphology shifts. Ancestral state reconstruction (ASR) of the two morphological types analysed in BiSSE was performed in *diversitree* using ML under the BiSSE model (ASR-BiSSE), thus accounting for differential

speciation, extinction and character transition rates in character optimization (Goldberg and Igić, 2008). To account for phylogenetic uncertainty, analyses were conducted separately for the MCC species tree and the same ten additional trees for which parameter values were estimated using MCMC-BiSSE. A higher number of trees was not analysed due to the high computational demands of this method. Given that the sister group to sect. *Versicolores* is currently unknown (Fernández-Mazuecos *et al.*, 2013), outgroup taxa, potentially biasing ASRs, were pruned from the analysed trees. Parameter distributions obtained in MCMC-BiSSE analyses were used to account for parameter uncertainty.

For comparison with the ASR-BiSSE approach described above, and to fully account for phylogenetic uncertainty, ASR was also performed using parsimony in Mesquite 2·75 (Maddison and Maddison, 2011). In this case, the three morphological types were included, thus treating Type II as a separate state. Reconstructions were conducted on the full set of species trees of the *BEAST posterior distribution using the 'trace character over trees' option. Additionally, the 'summarize state changes over trees' option was used to summarize the number of changes between character states across the Bayesian posterior distribution of trees.

Models of spur length and tube width evolution. We tested for the existence of one or more evolutionary optima for spur length and tube width using the ML method implemented in the R package *ouch* (Butler and King, 2004; King and Butler, 2009). Two models based on an Ornstein–Uhlenbeck process (Hansen, 1997) with one and two optima respectively were tested against a null Brownian motion model. Morphological Types I/II and III were defined as hypothetical selective regimes, and ancestral states were included based on the ASR-BiSSE reconstruction. Model comparisons were performed using AIC values. Analyses were conducted for the MCC species tree and the ten randomly sampled trees.

Pollinator observations

We performed flower visitor surveys in populations of the 12 Iberian species and subspecies of *Linaria* sect. *Versicolores*, which represent the full range of corolla shapes of the group. A total of 4618 min of observations (267–941 min per taxon) were performed in 2009, 2010, and 2011 in 14 populations that were also included in geometric morphometric and phylogenetic analyses. Visits were considered legitimate when the visitor touched the anthers and stigma. The placement of pollen on the insect body (thorax or proboscis) was recorded.

RESULTS

Phylogenetic relationships and divergence times

The ITS and ptDNA datasets had total aligned lengths of 610 and 2679 bp respectively (see characteristics of the four DNA regions in Table 2). Overall, the BI, ML and MP analyses yielded congruent topologies, except for some weakly supported clades. The separate phylogenetic analyses of nuclear ITS (Fig. 3A) and ptDNA (Fig. 3B) sequences consistently retrieved sect. *Versicolores* as a monophyletic group with strong statistical support [PP = 1; bootstrap support (BS) > 90 % in ML and MP analyses] and subsections *Elegantes* and *Versicolores* also as monophyletic (PP = 1; BS > 95 %) and sister to each other. However, topological incongruence between both datasets was extensive within subsect. *Versicolores*. In general, higher clade support values were obtained in ptDNA analyses.

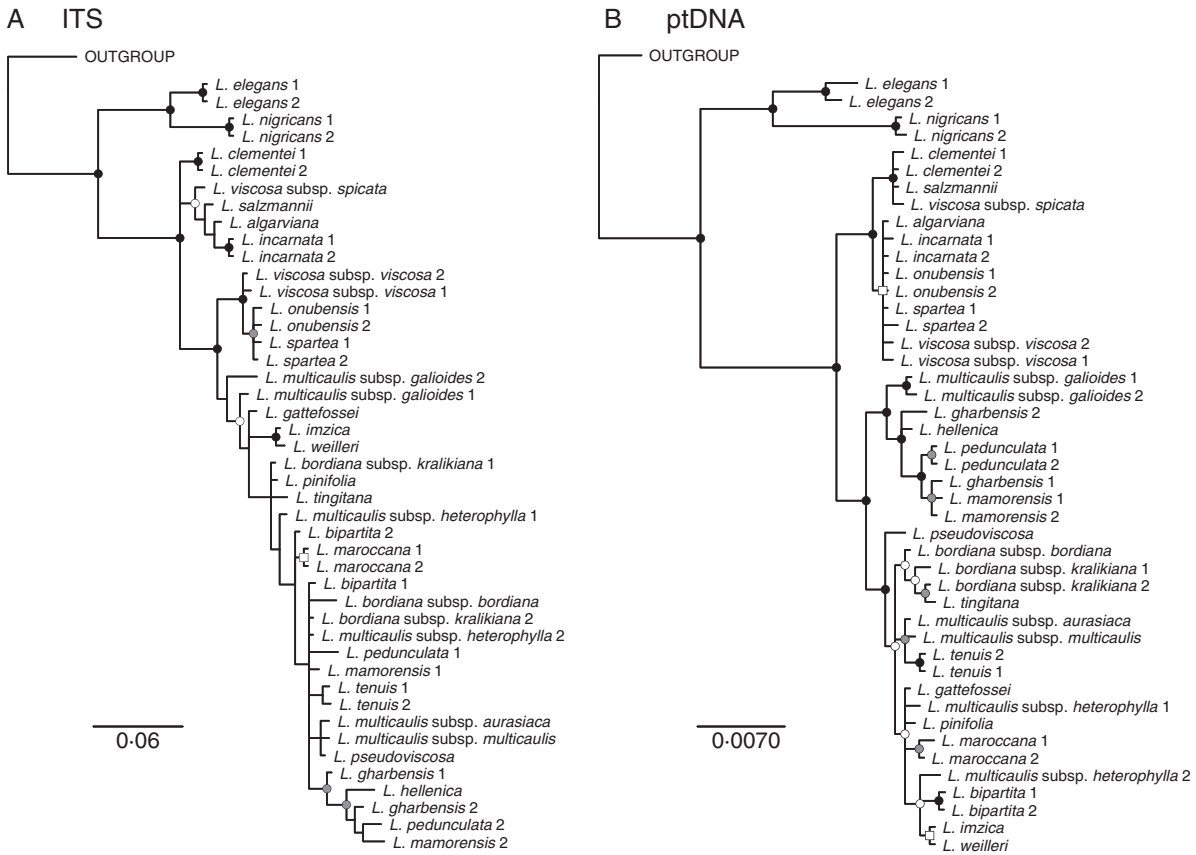
Separate dating analyses of the two loci (Supplementary Data Fig. S2) consistently recovered a crown age for sect. *Versicolores* around 6 Ma. This age was then used to calibrate the species tree analysis. The time-calibrated species tree obtained in *BEAST (Fig. 3C) strongly supported major clades of *Linaria* sect. *Versicolores*, but displayed lower resolution at shallow phylogenetic levels. Divergence between subsections *Elegantes* (PP = 1) and *Versicolores* (PP = 1) was dated back to the late Miocene or Pliocene. Two strongly supported sister clades were recognized within subsect. *Versicolores* (Fig. 3C): the 'Iberian clade' (PP = 1) included all species that are endemic or subendemic to the Iberian Peninsula, while the 'northern African clade' (PP = 1) included all northern African endemics, plus the Ibero-North African *L. pedunculata* and *L. gharbensis*, the southern Italian *L. multicaulis* subsp. *multicaulis* and the Greek *L. hellenica*. A Quaternary diversification of subsect. *Versicolores* was estimated, with the Iberian and northern African clades diverging 0·69–2·24 Ma and both clades diversifying in the last million years. The LTT plots (Fig. 3C) clearly depicted diversification of *Linaria* sect. *Versicolores* since the late Miocene–Pliocene, and primarily during the Quaternary.

Analysis of corolla shape

Measures of spur length and tube width from herbarium specimens of the 30 recognized species and subspecies of sect.

TABLE 2. *Characteristics of the four DNA regions sequenced for 45 individuals of* Linaria *sect.* Versicolores *and 11 outgroup taxa, and employed in phylogenetic analyses*

		Plastid DNA		
	Nuclear DNA, ITS (ITS1-5·8S-ITS2)	*rpl*32-*trn*L^{UAG}	*trn*K-*mat*K	*trn*S-*trn*G
Aligned length (bp)	610	830	1230	619
Ungapped length range (bp)	577–597	582–768	1206–1221	466–590
Pairwise identity (%)	90·7	94·0	98·2	92·7
Variable characters	199	211	186	139
Parsimony-informative characters	134	106	82	59
Mean G + C content (%)	56·1	23·6	32·3	27·4
Substitution model	GTR + G	GTR	GTR + G	HKI + I + G



Versicolores (Fig. 4A; Supplementary Data Table S3) revealed three major morphological types. Type I was the most frequent (22 species and subspecies) and was characterized by a broad tube (>2 mm) and wide variation in spur length (4–18 mm). Type II was only found in *L. clementei*, and displayed a broad tube (4–7 mm) and a very short spur (1–4·5 mm). Type III, found in seven taxa, had a narrow tube (1–3 mm) and variable spur length (5–19 mm). The same three groups were consistently recovered when measuring living specimens of the 12 taxa present in the Iberian Peninsula (Supplementary Data Fig. S3).

The canonical variate analysis of landmark-based geometric morphometric data of these 12 Iberian taxa (Fig. 4B) recovered the same three morphological types. The first two canonical variates explained 83·6 % of the variance, and all between-taxa Procrustes distances were statistically significant ($P < 0·001$) based on randomization tests. Variation along canonical variate 1 was related to palate development, since it affected the relative position and shape of the upper and lower lips (Fig. 4B; Supplementary Data Fig. S4A). Variation along canonical variate 2 was related to the shape of the spur and the relative sizes and positions of the upper and lower lips (Fig. 4B; Supplementary Data Fig. S4B). Taxa of Types I and II displayed well-developed palates, while Type III had broader and expanded lower lips. Type II (*L. clementei*) was clearly related to Type I, from which it was mainly differentiated by its short spur.

Evolution of flower morphology

An effect of flower morphology on diversification rates was supported by the likelihood ratio tests of BiSSE models (Table 3). In particular, we detected a significant effect on speciation rates, as the 'symmetric speciation' model was rejected for the MCC species tree and nine out of ten randomly chosen trees. Bayesian estimation of BiSSE parameters revealed significantly higher speciation rates for morphological Type I/II than for Type III, as shown by the non-overlapping 95 % credibility intervals obtained when analysing the MCC species tree (Fig. 5A) and the ten randomly chosen trees (Supplementary Data Fig. S5). No effect on extinction rates was detected (see the widely overlapping 95 % credibility intervals in Fig. 5A and Supplementary Data Fig. S5). Diversification (speciation minus extinction) rates were higher for Type I/II, although with certain overlap of the 95 % credibility intervals for three out of ten trees. No significant difference between transition rates was found.

Ancestral state reconstruction under state-dependent diversification (ASR-BiSSE) using the MCC species tree (Fig. 5B) recovered morphological Type III as ancestral to sect. *Versicolores* and subsect. *Elegantes*. Type I/II was inferred as ancestral to subsect. *Versicolores*, which implied an old shift from Type III to Type I/II. Four to five shifts from Type I/II to Type III were inferred within subsect. *Versicolores*, in both the Iberian and the northern African clade. Similar results were obtained when conducting ASR-BiSSE analysis on ten randomly chosen trees, although with variable ancestral state probabilities (Supplementary Data Fig. S6). Parsimony-based reconstructions yielded congruent results, although with equivocal reconstruction at the root node (Supplementary Data Fig. S7). Accordingly, zero to two shifts from Type III to Type I were estimated when accounting for topological uncertainty (Fig. 5C). Four to six shifts from Type I to Type III were estimated, and one shift from Type I to Type II was unequivocally reconstructed. No shifts were obtained from Type II to Types I and III, and from Type III to Type II (Fig. 5C).

Different models of trait evolution were supported for spur length and tube width in relation to the two main morphological types (Table 4). When analysing the MCC species tree, the Ornstein–Uhlenbeck model with one optimum at 8·98 mm was supported for spur length, while the Ornstein–Uhlenbeck model with two optima at 4·67 (Type I/II) and 1·86 mm (Type III) was preferred for tube width. Similar results were obtained for the ten additionally analysed trees (Table 4).

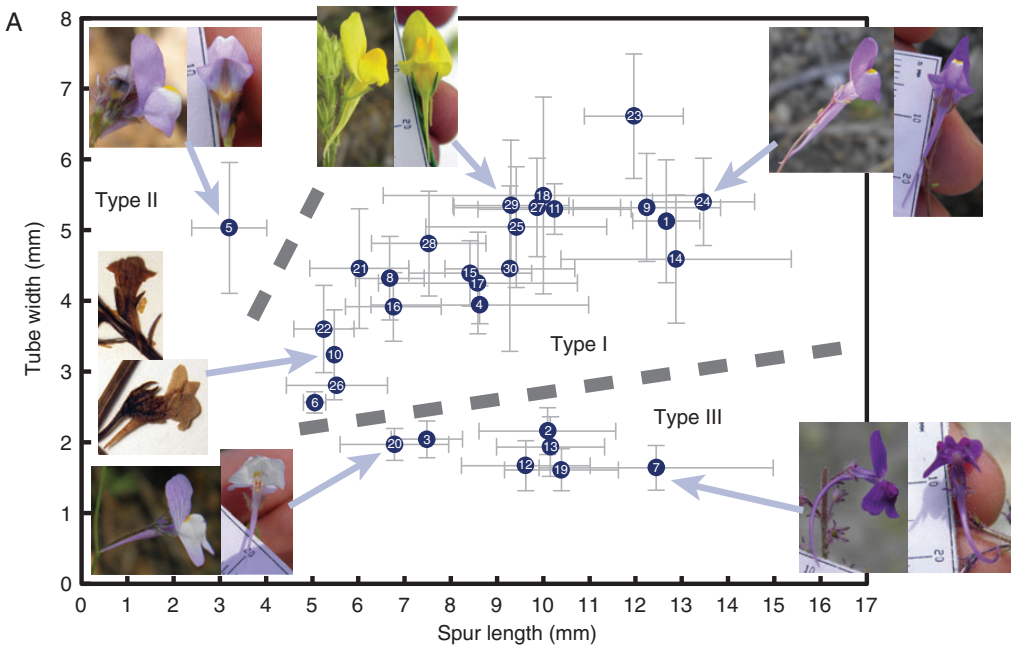
Pollinator observations

Observations of flower visitors in the 12 Iberian species and subspecies of *Linaria* sect. *Versicolores* (Table 5; Fig. 6) suggested that flowers of Types I and II are mainly pollinated by bees (Hymenoptera) carrying pollen on the back of the thorax (Fig. 6A), although sporadic visits by nectar-feeding butterflies (Lepidoptera; Fig. 6B) and bee flies (Bombyliidae, Diptera) carrying pollen on the proboscis were also recorded for three taxa: *L. spartea*, *L. viscosa* subsp. *viscosa* (Type I) and *L. clementei* (Type II). For the four Type III species, a wide variety of flower visitors were observed, most of them displaying a long proboscis: hawk moths (Sphingidae, Lepidoptera), *Anthophora*-like bees (Anthophorini, Apidae, Hymenoptera) and bee flies (Bombyliidae, Diptera). All of them carried pollen on the proboscis (Fig. 6C).

DISCUSSION

This study provides key insights into the evolution and diversification of bifid toadflaxes, with important consequences for understanding the relationship between floral specialization and species diversification. Species with narrow-tubed flowers were found to have evolved recurrently from broad-tubed ancestors, suggesting that similar selective pressures have driven flower evolution in independent lineages. However, the increasing pollinator specialization associated with narrower corolla tubes appears to have prevented narrow-tubed lineages from further diversification. Therefore, our results support a significant effect of floral specialization on the evolutionary success of flowering plant lineages.

FIG. 3. Phylogenetic analyses of *Linaria* sect. *Versicolores*. (A, B) Gene trees of ITS (A) and ptDNA (B) sequences. The 50 % majority rule consensus trees obtained in Bayesian analyses are shown. A black dot indicates clade support in BI (PP > 0·95), ML (ML – BS > 70 %) and MP (MP – BS > 70 %) analyses. A grey dot indicates support only in BI and ML analyses. A white dot indicates support only in the BI analysis. A white square indicates support only in the ML analysis. (C) Time-calibrated maximum clade credibility species tree obtained in the Bayesian *BEAST analysis based on ITS and ptDNA sequences. Node bars represent the 95 % highest posterior density intervals for the divergence time estimates. Numbers along the branches are Bayesian posterior probabilities. Major clades are named, including subsections following Sutton (1988). The inset shows a log-lineage-through-time plot for *Linaria* sect. *Versicolores*, based on 1000 trees randomly sampled from the posterior distribution of the *BEAST analysis. The thick line corresponds to the MCC species tree.



1 *L. algarviana*
2 *L. bipartita*
3 *L. bordiana* subsp. *bordiana*
4 *L. bordiana* subsp. *kralikiana*
5 *L. clementei*
6 *L. dissita*
7 *L. elegans*
8 *L. gattefossei*
9 *L. gharbensis*
10 *L. hellenica*
11 *L. imzica*
12 *L. incarnata*
13 *L. mamorensis*
14 *L. maroccana*
15 *L. multicaulis* subsp. *aurasiaca*
16 *L. multicaulis* subsp. *galioides*
17 *L. multicaulis* subsp. *heterophylla*
18 *L. multicaulis* subsp. *multicaulis*
19 *L. nigricans*
20 *L. onubensis*
21 *L. pedunculata*
22 *L. pinifolia*
23 *L. pseudoviscosa*
24 *L. salzmannii*
25 *L. spartea*
26 *L. tenuis*
27 *L. tingitana*
28 *L. viscosa* subsp. *spicata*
29 *L. viscosa* subsp. *viscosa*
30 *L. weilleri*

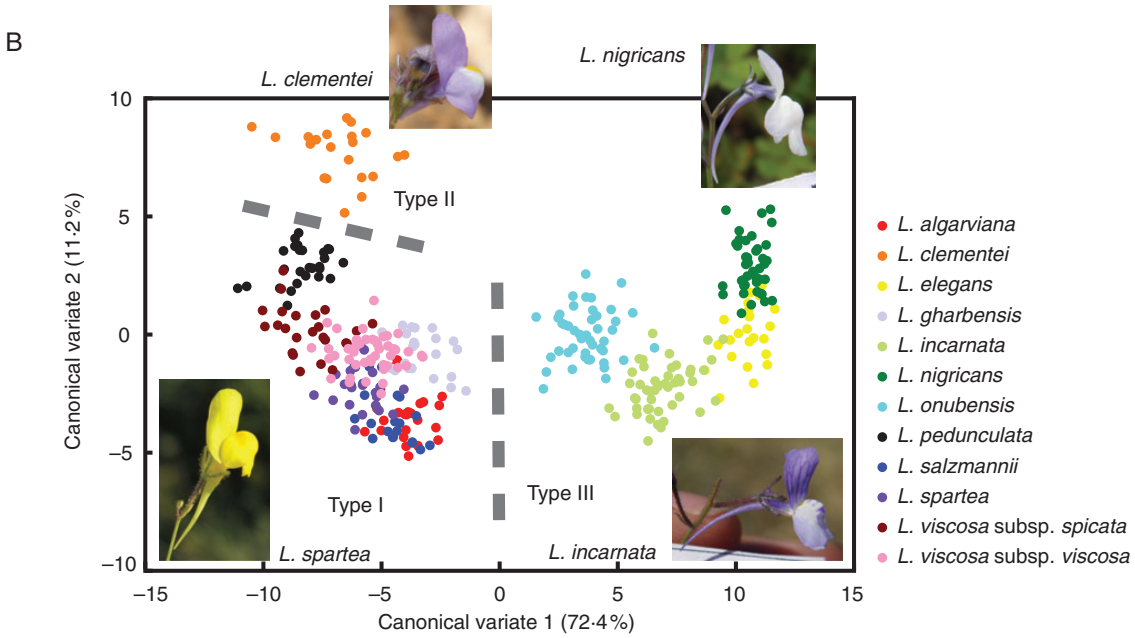


FIG. 4. Morphological traits of *Linaria* sect. *Versicolores* flowers. (A) Scatter plot of tube width versus spur length measured in 696 herbarium specimens representing the 30 species and subspecies of *Linaria* sect. *Versicolores*. Means (numbered dots) and standard deviations (bars) for each taxon are plotted. Notice the broad tubes of Types I and II, and the narrow tubes of Type III. (B) Canonical variate analysis of the landmark-based geometric morphometric dataset. Scatter plot of canonical variate 2 versus canonical variate 1 for 369 living specimens sampled in 18 populations belonging to the 12 Iberian species and subspecies (colours), which represent the full range of corolla shapes of *Linaria* sect. *Versicolores*. The variance explained by each axis is shown in brackets. In both plots, the three major morphological types discussed in the text are indicated, and representative taxa are shown.

TABLE 3. *Maximum likelihood estimates of BiSSE parameters and likelihood ratio tests of alternative models based on the maximum clade credibility tree of the *BEAST analysis*

	df	$\lambda_{I/II}$	λ_{III}	$\mu_{I/II}$	μ_{III}	$q_{I/II \rightarrow III}$	$q_{III \rightarrow I/II}$	lnL	AIC	χ^2	*P* value	No. trees with $P < 0.05$
Unconstrained model	6	3·75	2.83×10^{-6}	3·02	2·33	1·03	7.51×10^{-6}	−23·05	58·10	—	—	—
Symmetric speciation ($\lambda_{I/II} = \lambda_{III}$)	5	2·97	2·97	1·17	6·45	2·05	5.25×10^{-8}	−25·54	61·08	4·97	0·03*	9/10
Symmetric extinction ($\mu_{I/II} = \mu_{III}$)	5	3·53	1.93×10^{-9}	2·61	2·61	1·19	1.55×10^{-7}	−23·09	56·19	0·09	0·77 ns	0/10
Symmetric transition rate ($q_{I/II \rightarrow III} = q_{III \rightarrow I/II}$)	5	4·20	4.45×10^{-9}	3·87	1·77	0·88	0·88	−23·88	57·75	1·65	0·20 ns	0/10

The last column shows the number of trees (out of a random sample of ten trees from the posterior distribution of the *BEAST analysis) where each model was significantly worse ($P < 0.05$) than the unconstrained model according to a likelihood ratio test. Abbreviations and symbols: df, degrees of freedom; λ, speciation rates; μ, extinction rates; q, character transition rates; lnL, log likelihood; AIC, Akaike information criterion.

*$0.01 < P < 0.05$; ns, not significant.

Recent diversification of bifid toadflaxes

Our phylogenetic analyses based on nuclear and plastid sequences confirmed the monophyly of *Linaria* sect. *Versicolores* (Fernández-Mazuecos and Vargas, 2011; Fernández-Mazuecos *et al.*, 2013). Dating results indicate that diversification of bifid toadflaxes began in the late Miocene or Pliocene. Most inferred cladogenetic events occurred since the onset of the Mediterranean climate (~3·2 Ma; Suc, 1984) and particularly during the Quaternary (i.e. the last 2·6 Ma), as previously suggested based on plastid markers alone (Fernández-Mazuecos and Vargas, 2011; Fiz-Palacios and Valcárcel, 2013). While our species tree analysis provided strong support for several clades within sect. *Versicolores* (particularly the two subsections; Fig. 3C), fine-scale relationships between species were mostly unresolved or poorly supported. Low phylogenetic resolution is a common feature of recent radiations (e.g. Hughes and Eastwood, 2006; Scherson *et al.*, 2008; Valente *et al.*, 2010*a*). It is well known that rapid diversification brings about processes, such as hybridization and incomplete lineage sorting, that cause incongruence between gene trees and therefore may obscure phylogenetic relationships (Degnan and Rosenberg, 2009). This has recently been demonstrated for a different clade of toadflaxes, *Linaria* sect. *Supinae* (Blanco-Pastor *et al.*, 2012). Methods based on the multi-species coalescent (such as the one implemented in *BEAST), rather than concatenated analyses, currently constitute the best approach for the inference of species phylogenies in the presence of incongruent gene trees, because they account for incomplete lineage sorting (Edwards, 2009; Leaché and Rannala, 2011). We cannot rule out hybridization as a source of incongruence between gene trees (Fig. 3A, B). However, all phylogenetic comparative methods currently available are tree-based (Nunn, 2011). Incorporation of hybridization will strengthen phylogenetic inference (Yu *et al.*, 2011, 2012), and the development of comparative methods able to deal with reticulate phylogenies will probably lead to the reconstruction of more realistic evolutionary scenarios in the future. At present, however, the assumption of a tree-like phylogeny must be made in order to test evolutionary hypotheses using available tools. To account for uncertainty about phylogenetic relationships at shallow levels (part of which might be due to hybridization), we performed all comparative analyses on the consensus species tree and a random sample of ten species trees from the posterior distribution of the *BEAST analysis. Congruence of results across such sample was interpreted as evidence for a strong evolutionary signal in spite of phylogenetic uncertainty (Huelsenbeck *et al.*, 2000).

Convergence in the evolution of flower shape

Different biotic interactions affecting *Linaria* flowers have been studied previously, including floral herbivory, nectar robbery and insect pollination (Arnold, 1982; Stout *et al.*, 2000; Newman and Thomson, 2005*a*, *b*; Sánchez-Lafuente, 2007). The last is likely the most relevant factor affecting the evolution of morphological traits studied here (Sánchez-Lafuente, 2007). Our phylogenetic comparative analyses suggest that the evolution of flower morphology in bifid toadflaxes has been dominated by shifts between two morphological types mainly differentiated by the width of the tube and the development of the palate (Figs 4 and 5). It is suggested (Table 5; Fig. 6) that these two types constitute divergent strategies of pollen placement on nectar-feeding insects (Armbruster *et al.*, 1994; Grant, 1994; Kay, 2006; Yang *et al.*, 2007). These two strategies of *Linaria* pollination (Fig. 6) were first identified by Robertson (1888) and Hill (1909). One strategy corresponds to the typically nototribic pollination of broad-tubed species (Type I/II), in which pollen is deposited on the back of the thorax (scutum) of the nectar-feeding insect (Fig. 6A). This is the strategy found in most *Linaria* species of other sections, and has been demonstrated to result in effective pollination (Macior, 1967; Arnold, 1982; Stout *et al.*, 2000; Newman and Thomson, 2005*a*; Sánchez-Lafuente, 2007; Sánchez-Lafuente *et al.*, 2011). In sect. *Versicolores*, the placement of the first optimum inferred by the two-peak Ornstein–Uhlenbeck model (~4·67 mm; Table 4) suggests an adaptive adjustment to the thorax width of frequent pollinators. Indeed, closely related species pollinated by similar insects should be similarly selected for the floral phenotype that most efficiently uses these pollinators (Kay and Sargent, 2009). In our case, an adjustment of flower tube size to pollinator size probably maximizes pollen transfer in personate, wide-tubed corollas (but see Vargas *et al.* (2010) for *Antirrhinum*). The other strategy is displayed by narrow-tubed species (Type III), in which nectar-feeding insects usually carry pollen on the proboscis (Fig. 6C). In this situation, pollen transfer is maximized by narrowing the tube, so that contact of the proboscis with the anthers and the stigma is guaranteed when the insect reaches for nectar contained in the spur (Robertson, 1888; Kampny, 1995). This would lead to the

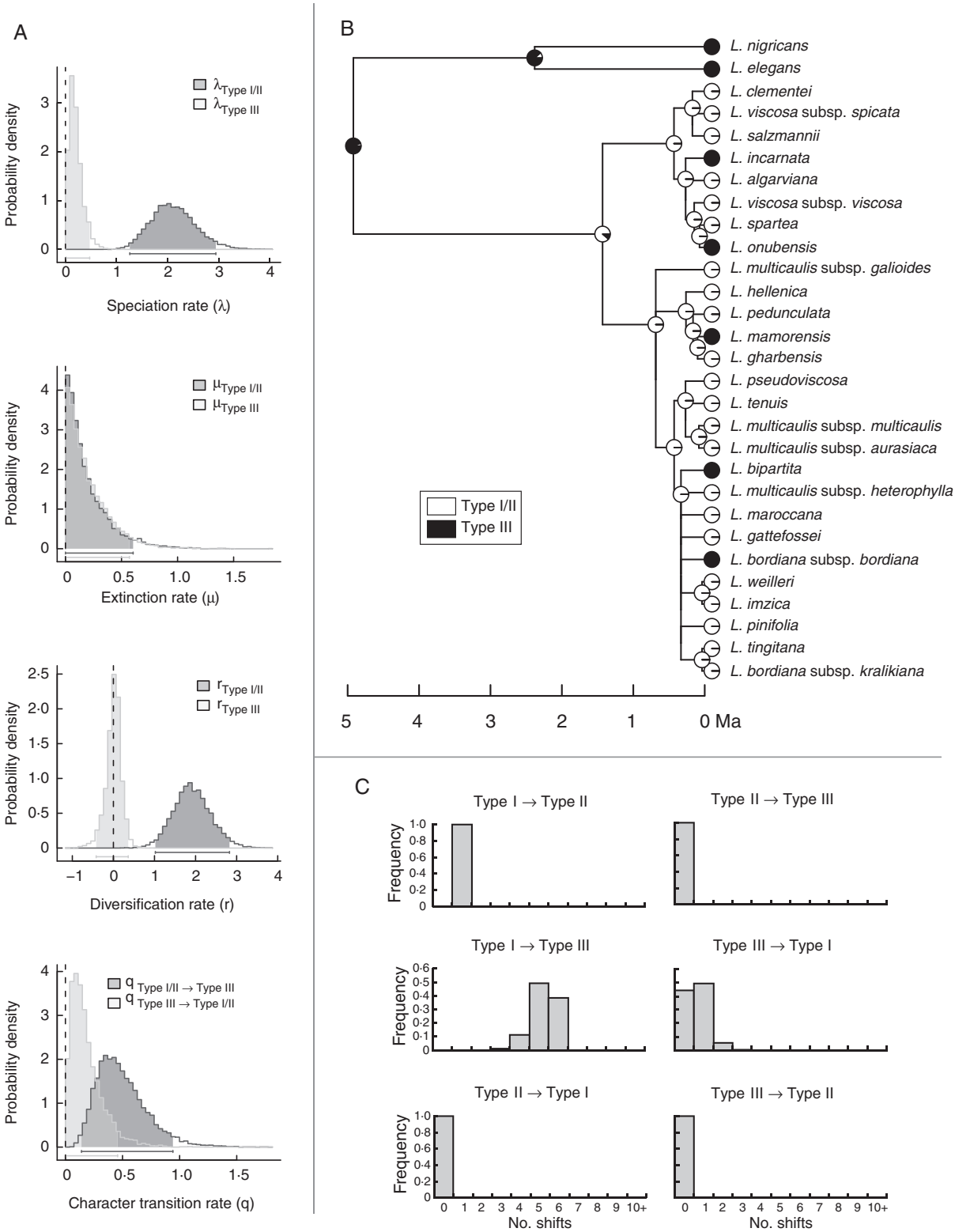


FIG. 5. Analyses of diversification rates and flower type transitions in *Linaria* sect. *Versicolores*. (A) Results of the binary-state speciation and extinction (BiSSE) analysis of the MCC species tree (Fig. 3C), considering two character states corresponding to morphological Types I/II (broad tube) and III (narrow tube). Posterior distributions of parameters (speciation rates, extinction rates, diversification rates and character transition rates) obtained in the MCMC-BiSSE analysis are shown. Horizontal bars indicate the 95 % credibility interval for each parameter. (B) Maximum likelihood ancestral state reconstruction of morphological Types I/II and III under state-dependent diversification (ASR-BiSSE), based on parameter estimates shown in A. The tree is the MCC species tree with nodes with PP $< 0{\cdot}5$ collapsed. (C) Summary distributions of the number of shifts between flower types inferred when implementing parsimony optimization over the full posterior distribution of trees obtained in the *BEAST analysis.

TABLE 4. *Testing of evolution models for spur length and tube width*

	lnL	AICc	σ^2	α	Optima (mm)	No. trees supporting the model
Spur length						
BM	−83·98	172·43	230·45	NA	NA	0/10
OU1*	−68·36	143·68	4891·41	374·84	8·98 (8·94–9·06)	10/10
OU2	−68·10	145·86	2253·02	175·54	8·77 (8·72–8·87) 9·61 (9·57–9·78)	0/10
Tube width						
BM	−62·33	129·12	51·76	NA	NA	0/0
OU1	−51·12	109·20	6301·93	1584·62	3·99 (3·81–4·00)	0/0
OU2*	−32·27	74·20	726·20	669·87	4·67 (4·66–4·68) 1·86 (1·83–1·86)	10/10

Three models were tested: Brownian motion (BM), Ornstein–Uhlenbeck process with one optimum (OU1) and Ornstein–Uhlenbeck process with two optima (OU2). Values for the MCC species tree obtained in the *BEAST analysis are shown. Optimum values for the MCC species tree and minimum and maximum values obtained for ten randomly chosen species trees from the Bayesian posterior distribution (in brackets) are indicated. The last column summarizes the number of trees of the same sample where each model was significantly supported based on corrected AICc values. Abbreviations and symbols: *preferred model; lnL, log likelihood; AICc, corrected Akaike information criterion; σ, α, model parameters.

TABLE 5. *Potential pollinators of Iberian species of* Linaria *sect.* Versicolores*, recorded after 4618 min of observations in 2009, 2010 and 2011*

Taxon	Pollinators
Subsect. *Versicolores*	
L. algarviana (I)	Hymenoptera: *Ceratina saundersi* (S) + Coleoptera: *Attagenus* sp. (S)
L. clementei (II)	Hymenoptera: *Amegilla quadrifasciata* (T), *Rhodanthidium sticticum* (T), *Bombus ruderatus* (T), *Xylocopa* sp. (T), *Heliophila bimaculata* (T) −, *Ceratina mocsaryi* (S) − Diptera: *Dischistus separatus* (P) Lepidoptera (P)
L. gharbensis (I)	Hymenoptera: *Anthophora plumipes* (T) +
L. incarnata (III)	Diptera: *Systoechus gradatus* (P), *Amictus variegatus* (P) Lepidoptera: *Thymelicus* spp. (P) Hymenoptera: *Lasioglossum* sp. (S), *Ceratina* sp. (S) −
L. onubensis (III)	Hymenoptera: *Eucera nigrilabris* (P) +
L. pedunculata (I)	Not seen (autogamous species)
L. salzmannii (I)	Hymenoptera: *Heliophila bimaculata* (T) +, *Rhodanthidium sticticum* (T), *Lasioglossum* sp. (S), *Hoplitis* sp. (S) −
L. spartea (I)	Hymenoptera: *Apis mellifera* (T), *Heliophila bimaculata* (T), *Ceratina cucurbitina* (S) −, *Lasioglossum* sp. (S) − Lepidoptera: *Euchloe crameri* (P) −
L. viscosa subsp. *viscosa* (I)	Hymenoptera: *Apis mellifera* (T) +, *Hoplitis* sp. (T), *Xylocopa uclesiensis* (T) −, *Heliophila bimaculata* (T) −, *Lasioglossum* sp. (S) − Lepidoptera: *Euchloe crameri* (P) −
L. viscosa subsp. *spicata* (I)	Hymenoptera: *Rhodanthidium sticticum* (T) +, *Osmia andrenoides* (T)
Subsect. *Elegantes*	
L. elegans (III)	Lepidoptera: *Macroglossum stellatarum* (P) +, *Lasiommata megera* (P) − Hymenoptera: *Anthophora retusa* (P) − Diptera: *Bombylius major* (P) −
L. nigricans (III)	Hymenoptera: *Eucera nigrilabris* (P) +, *Apis mellifera* (P) Lepidoptera: *Colias croceus* (P) −, *Pontia daplidice* (P) −

Observed strategies of pollen placement on insect body are coded as follows: (T) pollen placed on the back of the thorax; (P) pollen placed on the proboscis; (S) small insects with variable pollen placement. +, >50 % of flower visits; −, <5 % of flower visits. Morphological types are indicated in brackets after taxon names.

second optimum of tube width (∼1·86 mm; Table 4), which is large enough to fit the anthers (∼0·5 mm) but narrow enough to guarantee pollen transfer by long-proboscis visitors. Similar strategies of pollen placement on pollinators are frequently found in angiosperm lineages with spurred flowers, particularly those pollinated by long-proboscis insects (Herrera, 1993; Johnson and Steiner, 1997; Schiestl and Schlüter, 2009). In *Linaria*, the narrow-tubed strategy has been previously related to pollination by butterflies (Robertson, 1888; Hill, 1909) and moths (Sutton, 1988; Kampny, 1995). Our observations partially support these predictions (see *L. elegans* and *L. incarnata* in Table 5). Additionally, we have found the narrow-tube morphology to be also suited to other long-proboscis pollinators, such as long-tongued bees (mainly from tribe Anthophorini) and bee flies (Bombyliidae) (Table 5; see Supplementary Data Appendix S2 for further discussion on flower visitors).

Several independent origins of similarly configured narrow-tubed flowers have been inferred by ancestral state reconstructions (Fig. 5B, C and Supplementary Data Figs S6 and S7). Therefore, this is definitely a case of phenotypic homoplasy, i.e. convergence *sensu* Scotland (2011). In addition, reversal to an ancestral state (which can be regarded as a special case of convergence; Scotland, 2011) is suggested by the ancestral narrow-tubed flower inferred by the ASR-BiSSE analysis, together with its loss and subsequent repeated reappearance in subsect. *Versicolores* (Fig. 5B) (Hall, 2003; Porter and Crandall, 2003). The convergent evolution of narrow-tubed phenotypes in several different lineages of bifid toadflaxes may have involved similar genetic and developmental mechanisms (Hall, 2012), in which case it would be interpreted as an instance of parallel evolution *sensu* Scotland (2011) (note that the definitions of convergence and parallelism are controversial; see also Hall, 2003; Arendt and Reznick, 2008; Wake *et al.*, 2011; Hall, 2012). Homoplasy of morphological traits can result from common adaptive responses to similar selection pressures, coupled with genetic and developmental constraints (Wake, 1991; Brakefield, 2006; Wake *et al.*, 2011). While no information about the latter is yet available for the study group, pollinator observations suggest an adaptive meaning of the two morphological types, as they correspond to different strategies of pollen placement on pollinators. Indeed, the recurrent shifts

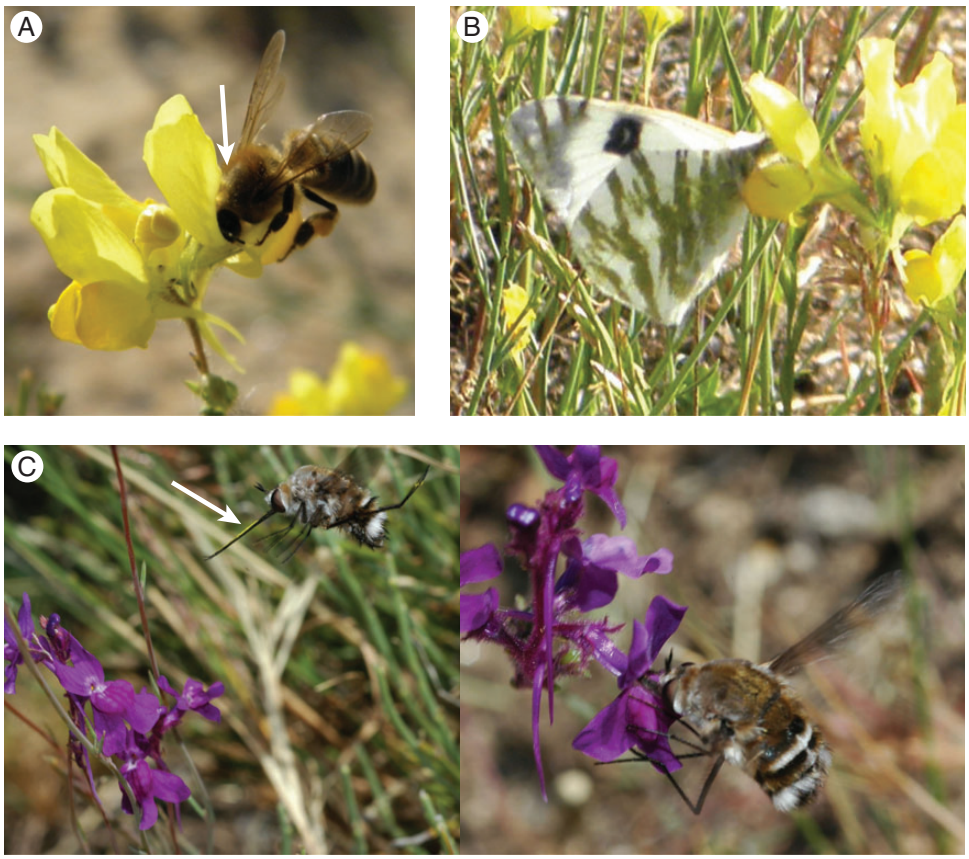


FIG. 6. Different behaviours of potential pollinators of morphological Types I (broad tube) and III (narrow tube). (A) *Apis mellifera* (Hymenoptera) in *L. viscosa* subsp. *viscosa* (Type I). (B) *Euchloe* sp. (Lepidoptera) in *L. viscosa* subsp. *viscosa*. (C) Bombyliidae (Diptera) in *L. elegans* (Type III). White arrows indicate pollen placement on pollinators. Photographs by M. Fernández-Mazuecos (A, B) and P. Vargas (C).

from broad-tubed flowers to narrow-tubed ones in subsect. *Versicolores* are not surprising given the fact that broad-tubed species are visited at a low rate by long-proboscis insects, including butterflies and even bee flies, which carry pollen on their proboscis (Table 5; Fig. 6B). Following the 'pollinator shift' hypothesis (Grant and Grant, 1965; Stebbins, 1970; Campbell, 2008), when a broad-tubed species is faced with an environment in which such long-proboscis insects are dominant, natural selection would favour a narrowing of the tube that would maximize pollen transfer, a mechanism similar to that previously invoked to explain spur elongation in American columbines (Whittall and Hodges, 2007). Additional research on reproductive and pollination biology at the population level will be needed to shed further light on the microevolutionary mechanisms (including selective pressures) involved in these putatively adaptive morphological shifts.

Trait dependent diversification?

Even though we have inferred a higher number of shifts from broad- to narrow-tubed flowers (4–6) than in the opposite direction (0–2) (Fig. 5B, C), a directional trend in the evolution of morphological types is not supported by BiSSE analyses (Fig. 5A and Supplementary Data Fig. S5). Instead, our analyses of trait-dependent diversification revealed a dissimilar evolutionary success of the broad- and narrow-tube strategies, as shown by the consistently higher speciation rate of broad-tubed species found in BiSSE analyses (Fig. 5A and Supplementary Data Fig. S5). Thus, despite having repeatedly evolved, the narrow-tubed strategy seems to display limited success in terms of speciation. In fact, radiation of subsect. *Versicolores* (23–28 species) may have been triggered not only by the onset of the Mediterranean climate (see above), but also by a shift from the ancestral narrow tube (as inferred by the ASR-BiSSE analysis; Fig. 5B) to the broad tube in the common ancestor of this clade. This is illustrated by the fact that the sister subsect. *Elegantes*, which maintained the ancestral state (narrow tube), yielded only two species during the same period of time and under similar climatic conditions (Fig. 3C; see Supplementary Data Appendix S2 for additional analyses and discussion).

BiSSE results suggest that trait-dependent diversification rates, rather than asymmetrical rates of change, are responsible for the contrasting species diversities of the two morphological types. Caution should be exercised in this interpretation, given the small size of our study group (Maddison *et al.*, 2007; Wertheim and Sanderson, 2011; Davis *et al.*, 2013). It is important to note that our analyses were able to reject the null hypothesis of symmetrical speciation rates (Table 3; Fig. 5A) despite the reported low statistical power of BiSSE with small datasets (Davis *et al.*, 2013). However, confounding effects of asymmetrical extinction rates and/or rates of change cannot be ruled out. In any case, the differential diversification success of morphological types is further suggested at the genus level (~150 species), as shown by the fact that additional *Linaria* clades

displaying narrow tubes are remarkably species-poor (sects *Macrocentrum* and *Lectoplectron*, six species) compared with broad-tubed clades (sects *Linaria*, *Speciosae*, *Supinae*, *Diffusae* and *Pelisserianae*, >120 species) (Sutton, 1988; Fernández-Mazuecos *et al.*, 2013).

Several flower traits have been found previously to influence diversification rates, including flower symmetry (Sargent, 2004), biotic/abiotic pollination mode (Dodd *et al.*, 1999) and the presence of nectar spurs (Hodges and Arnold, 1995; Hodges, 1997; but see Hagen and Kadereit, 2003; Cacho *et al.*, 2010). In general, it has been proposed that floral specialization promotes diversification (but see Smith *et al.*, 2008), although it has in turn been suggested that high species diversity may promote floral specialization (Armbruster and Muchhala, 2009). Our results suggest that traits restricting pollinator access to rewards and pollen placement on pollinators have significant effects on diversification rates of *Linaria* sect. *Versicolores*. Mechanisms of species selection (Stanley, 1975; Jablonski, 2008; FitzJohn, 2010; Rabosky and McCune, 2010) causing such differences in trait-dependent 'emergent fitness' (i.e. heritable differences in net diversification rates) are different from those involved in selection at the individual level (Rabosky and McCune, 2010). Indeed, the narrow-tube strategy in bifid toadflaxes has recurrently evolved by means of individual-level selection mechanisms, yet it may have exerted a negative influence on diversification rates, thus leading to the low frequency of this character state (Fig. 5). Specific mechanisms of species selection acting in bifid toadflaxes may include differential opportunities for exploitation of pollinator fauna (e.g. higher diversity of pollinators carrying pollen on the thorax than on the proboscis) and differential extinction risks (e.g. due to the likely higher specialization of narrow-tubed species; Johnson and Steiner, 2000). Even though our analyses have detected significant effects on speciation rather than extinction rates, the latter cannot be ruled out given the reported limitations of phylogeny-based methods to detect variation in extinction rates (FitzJohn, 2010; Rabosky, 2010). Further research will be needed to understand the mechanisms that account for the effects of pollinator-restrictive traits on diversification rates of *Linaria* and other genera with highly specialized corollas.

Our results support a relationship between resource specialization and evolutionary success in terms of diversification. The intriguing finding that opposing individual-level and species-level selection pressures may drive the evolution of specialized traits is worth further investigation in additional model systems.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Appendix S1: Herbarium specimens studied for taxonomic delimitation and measurement of flower traits. Appendix S2: Additional methods, results and discussion. Fig. S1: Bayesian phylogenetic analysis of ITS haplotypes inferred by PHASE. Fig. S2: Maximum clade credibility trees produced by relaxed molecular-clock analyses of ITS and ptDNA sequences in BEAST. Fig. S3: Scatter plot of tube width versus spur length measured from living specimens of the 12 Iberian species and subspecies of *Linaria* sect. *Versicolores*. Fig. S4: Visualization of landmark displacements along canonical variates 1 and 2 of the geometric morphometric analysis. Fig. S5: Results of the BiSSE analysis of ten species trees randomly chosen from the posterior distribution of the *BEAST analysis. Fig. S6: Ancestral state reconstructions of morphological Types I/II and III under state-dependent diversification, performed on ten trees randomly chosen from the posterior distribution of the *BEAST analysis. Fig. S7: Summary of parsimony-based ancestral state reconstruction of the three major flower types of *Linaria* sect. *Versicolores*, conducted over the full posterior distribution of trees obtained in the *BEAST analysis. Table S1: Voucher specimens and GenBank accession numbers of species and subspecies of *Linaria* sect. *Versicolores* and the outgroup sampled for DNA sequencing. Table S2: Voucher specimens of Iberian species and subspecies of *Linaria* sect. *Versicolores* and the outgroup sampled for geometric morphometric analyses. Table S3: Measures of spur length and tube width obtained from herbarium specimens of the 30 species and subspecies of *Linaria* sect. *Versicolores*.

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LITERATURE CITED

Abdelaziz M, Lorite J, Muñoz-Pajares AJ, Herrador MB, Perfectti F, Gómez JM. 2011. Using complementary techniques to distinguish cryptic species: a new *Erysimum* (Brassicaceae) species from North Africa. *American Journal of Botany* **98**: 1049–1060.

Abràmoff MD, Magalhães PJ, Ram SJ. 2004. Image processing with ImageJ. *Biophotonics International* **11**: 36–42.

Albrecht GH. 1980. Multivariate analysis and the study of form, with special reference to canonical variate analysis. *American Zoologist* **20**: 679–693.

Alexandersson R, Johnson SD. 2002. Pollinator-mediated selection on flower-tube length in a hawkmoth-pollinated *Gladiolus* (Iridaceae). *Proceedings of the Royal Society Series B, Biological Sciences* **269**: 631–636.

Arendt J, Reznick D. 2008. Convergence and parallelism reconsidered: what have we learned about the genetics of adaptation? *Trends in Ecology & Evolution* **23**: 26–32.

Armbruster WS, Baldwin BG. 1998. Switch from specialized to generalized pollination. *Nature* **394**: 632–632.

Armbruster WS, Muchhala N. 2009. Associations between floral specialization and species diversity: cause, effect, or correlation? *Evolutionary Ecology* **23**: 159–179.

Armbruster WS, Edwards ME, Debevec EM. 1994. Floral character displacement generates assemblage structure of Western Australian triggerplants (*Stylidium*). *Ecology* **75**: 315–329.

Armbruster WS, Lee J, Baldwin BG. 2009. Macroevolutionary patterns of defense and pollination in *Dalechampia* vines: adaptation, exaptation, and evolutionary novelty. *Proceedings of the National Academy of Sciences of the USA* **106**: 18085–18090.

Arnold RM. 1982. Pollination, predation and seed set in *Linaria vulgaris* (Scrophulariaceae). *American Midland Naturalist* **107**: 360–369.

Bell CD, Soltis DE, Soltis PS. 2010. The age and diversification of the angiosperms re-revisited. *American Journal of Botany* **97**: 1296–1303.

Blanco-Pastor JL, Vargas P, Pfeil BE. 2012. Coalescent simulations reveal hybridization and incomplete lineage sorting in Mediterranean *Linaria*. *PLoS One* **7**: e39089.

Blanco-Pastor JL, Fernández-Mazuecos M, Vargas P. 2013. Past and future demographic dynamics of alpine species: limited genetic consequences despite dramatic range contraction in a plant from the Spanish Sierra Nevada. *Molecular Ecology* **22**: 4177–4195.

Brakefield PM. 2006. Evo-devo and constraints on selection. *Trends in Ecology & Evolution* **21**: 362–368.

Bruun HG. 1937. Genetical notes on *Linaria*, I-II. *Hereditas* **22**: 395–400.

Butler MA, King AA. 2004. Phylogenetic comparative analysis: a modeling approach for adaptive evolution. *American Naturalist* **164**: 683–695.

Cacho NI, Berry PE, Olson ME, Steinmann VW, Baum DA. 2010. Are spurred cyathia a key innovation? Molecular systematics and trait evolution in the slipper spurges (*Pedilanthus* clade: *Euphorbia*, Euphorbiaceae). *American Journal of Botany* **97**: 493–510.

Campbell DR. 2008. Pollinator shifts and the origin and loss of plant species. *Annals of the Missouri Botanical Garden* **95**: 264–274.

Carrió E, Güemes J, Herreros R. 2012. Pollination biology in an endangered rocky mountain toadflax (*Linaria cavanillesii*). *Plant Biosystems* **147**: 354–363.

Darwin C. 1862. *On the various contrivances by which British and foreign orchids are fertilized by insects*. London: John Murray.

Darwin C. 1876. *The effects of cross and self fertilisation in the vegetable kingdom*. London: John Murray.

Darwin C. 1877. *The different forms of flowers on plants of the same species*. London: John Murray.

Davis MP, Midford PE, Maddison W. 2013. Exploring power and parameter estimation of the BiSSE method for analyzing species diversification. *BMC Evolutionary Biology* **13**: 38.

Degnan JH, Rosenberg NA. 2009. Gene tree discordance, phylogenetic inference and the multispecies coalescent. *Trends in Ecology & Evolution* **24**: 332–340.

Dobignard A. 1997. Nouvelles observations sur la flore du Maroc. 3. *Candollea* **52**: 119–157.

Docherty Z. 1982. Self-incompatibility in *Linaria*. *Heredity* **49**: 349–352.

Dodd ME, Silvertown J, Chase MW. 1999. Phylogenetic analysis of trait evolution and species diversity variation among angiosperm families. *Evolution* **53**: 732–744.

Drummond AJ, Rambaut A. 2007. BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology* 7: 214.

Drummond AJ, Ho SYW, Phillips MJ, Rambaut A. 2006. Relaxed phylogenetics and dating with confidence. *PLoS Biology* **4**: 699–710.

Edwards SV. 2009. Is a new and general theory of molecular systematics emerging? *Evolution* **63**: 1–19.

Endress PK. 1992. Evolution and floral diversity: the phylogenetic surroundings of *Arabidopsis* and *Antirrhinum*. *International Journal of Plant Sciences* **153**: S106–S122.

Endress PK. 1994. *Diversity and evolutionary biology of tropical flowers*. Cambridge: Cambridge University Press.

Faegri K, van der Pijl L. 1979. *The principles of pollination ecology*. Oxford: Pergamon.

Fennane M, Ibn Tattou M. 1998. Catalogue des plantes vasculaires rares, menacées ou endémiques du Maroc. *Bocconea* **8**: 1–243.

Fenster CB, Armbruster WS, Wilson P, Dudash MR, Thomson JD. 2004. Pollination syndromes and floral specialization. *Annual Review of Ecology, Evolution, and Systematics* **35**: 375–403.

Fernández-Mazuecos M, Vargas P. 2011. Historical isolation *versus* recent long-distance connections between Europe and Africa in bifid toadflaxes (*Linaria* sect. *Versicolores*). *PLoS One* **6**: e22234.

Fernández-Mazuecos M, Vargas P. 2013. Congruence between distribution modelling and phylogeographical analyses reveals Quaternary survival of a toadflax species (*Linaria elegans*) in oceanic climate areas of a mountain ring range. *New Phytologist* **198**: 1274–1289.

Fernández-Mazuecos M, Blanco-Pastor JL, Vargas P. 2013. A phylogeny of toadflaxes (*Linaria* Mill.) based on nuclear internal transcribed spacer sequences: systematic and evolutionary consequences. *International Journal of Plant Sciences* **174**: 234–249.

FitzJohn RG. 2010. Quantitative traits and diversification. *Systematic Biology* **59**: 619–633.

FitzJohn RG. 2012. Diversitree: comparative phylogenetic analyses of diversification in R. *Methods in Ecology and Evolution* **3**: 1084–1092.

FitzJohn RG, Maddison WP, Otto SP. 2009. Estimating trait-dependent speciation and extinction rates from incompletely resolved phylogenies. *Systematic Biology* **58**: 595–611.

Fiz-Palacios O, Valcárcel V. 2013. From Messinian crisis to Mediterranean climate: a temporal gap of diversification recovered from multiple plant phylogenies. *Perspectives in Plant Ecology, Evolution and Systematics* **15**: 130–137.

Fleming TH, Geiselman C, Kress WJ. 2009. The evolution of bat pollination: a phylogenetic perspective. *Annals of Botany* **104**: 1017–1043.

Futuyma DJ, Moreno G. 1988. The evolution of ecological specialization. *Annual Review of Ecology and Systematics* **19**: 207–233.

Gallagher MS. 2010. JSTOR Plant Science. In: Nimis PL, Lebbe RV. eds. *Tools for identifying biodiversity: progress and problems*. Trieste: Edizioni Università di Trieste, 417–418.

Gernhard T. 2008. The conditioned reconstructed process. *Journal of Theoretical Biology* **253**: 769–778.

Goldberg EE, Igić B. 2008. On phylogenetic tests of irreversible evolution. *Evolution* **62**: 2727–2741.

Goloboff P, Farris J, Nixon K. 2003. TNT: tree analysis using new technology. http://www.zmuc.dk/public/phylogeny/tnt/ (last accessed 24 April 2013).

Gómez JM, Zamora R. 2006. Ecological factors that promote the evolution of generalization in pollination systems. In: Waser N, Ollerton J. eds. *Plant-pollinator interactions: from generalization to specialization*. Chicago: University of Chicago Press, 145–166.

Gómez JM, Perfectti F, Camacho JPM. 2006. Natural selection on *Erysimum mediohispanicum* flower shape: insights into the evolution of zygomorphy. *American Naturalist* **168**: 531–545.

Gómiz F. 2004. Dos novedades de Marruecos: *Linaria imzica* Gómiz, *sp. nov.* (Scrophulariaceae) y *Centaurea austromaroccana* (Förther & Podlech) Gómiz, *comb. & stat. nov.* (Compositae). *Anales del Jardín Botánico de Madrid* **61**: 205–208.

Grant V. 1949. Pollination systems as isolating mechanisms in angiosperms. *Evolution* **3**: 82–97.

Grant V. 1994. Modes and origins of mechanical and ethological isolation in angiosperms. *Proceedings of the National Academy of Sciences of the USA* **91**: 3–11.

Grant V, Grant KA. 1965. *Flower pollination in the phlox family*. New York: Columbia University Press.

Hagen KB, Kadereit JW. 2003. The diversification of *Halenia* (Gentianaceae): ecological opportunity versus key innovation. *Evolution* **57**: 2507–2518.

Hall BK. 2003. Descent with modification: the unity underlying homology and homoplasy as seen through an analysis of development and evolution. *Biological Reviews* **78**: 409–433.

Hall BK. 2012. Parallelism, deep homology, and evo-devo. *Evolution & Development* **14**: 29–33.

Hamdi SMM, Assadi M, Vaezi G, Mirtadzadini M. 2009. A new species of *Linaria* sect. *Versicolores* (Scrophulariaceae) from Iran. *Novon* **18**: 340–343.

Hansen TF. 1997. Stabilizing selection and the comparative analysis of adaptation. *Evolution* **51**: 1341–1351.

Heled J, Drummond AJ. 2010. Bayesian inference of species trees from multilocus data. *Molecular Biology and Evolution* **27**: 570–580.

Herrera CM. 1990. The adaptedness of the floral phenotype in a relict endemic, hawkmoth-pollinated violet. 1. Reproductive correlates of floral variation. *Biological Journal of the Linnean Society* **40**: 263–274.

Herrera CM. 1993. Selection on floral morphology and environmental determinants of fecundity in a hawk moth-pollinated violet. *Ecological Monographs* **63**: 251–275.

Hill EJ. 1909. Pollination in *Linaria* with special reference to cleistogamy. *Botanical Gazette* **47**: 454–466.
Hodges SA. 1997. Floral nectar spurs and diversification. *International Journal of Plant Sciences* **158**: 81–88.
Hodges SA, Arnold ML. 1995. Spurring plant diversification: are floral nectar spurs a key innovation? *Proceedings of the Royal Society Series B, Biological Sciences* **262**: 343–348.
Huelsenbeck JP, Rannala B, Masly JP. 2000. Accommodating phylogenetic uncertainty in evolutionary studies. *Science* **288**: 2349–2350.
Hughes C, Eastwood R. 2006. Island radiation on a continental scale: exceptional rates of plant diversification after uplift of the Andes. *Proceedings of the National Academy of Sciences of the USA* **103**: 10334–10339.
Jablonski D. 2008. Species selection: theory and data. *Annual Review of Ecology, Evolution, and Systematics* **39**: 501–524.
Johnson SD, Steiner KE. 1997. Long-tongued fly pollination and evolution of floral spur length in the *Disa draconis* complex (Orchidaceae). *Evolution* **51**: 45–53.
Johnson SD, Steiner KE. 2000. Generalization versus specialization in plant pollination systems. *Trends in Ecology & Evolution* **15**: 140–143.
Kampny CM. 1995. Pollination and flower diversity in Scrophulariaceae. *Botanical Review* **61**: 350–366.
Katoh K, Misawa K, Kuma K, Miyata T. 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research* **30**: 3059–3066.
Kay KM. 2006. Reproductive isolation between two closely related hummingbird pollinated neotropical gingers. *Evolution* **60**: 538–552.
Kay KM, Sargent RD. 2009. The role of animal pollination in plant speciation: integrating ecology, geography, and genetics. *Annual Review of Ecology, Evolution, and Systematics* **40**: 637–656.
King AA, Butler MA. 2009. ouch: Ornstein-Uhlenbeck models for phylogenetic comparative hypotheses (R package). http://ouch.r-forge.r-project.org (last accessed 24 April 2013).
Klingenberg CP. 2011. MorphoJ: an integrated software package for geometric morphometrics. *Molecular Ecology Resources* **11**: 353–357.
Klingenberg CP, Monteiro LR. 2005. Distances and directions in multidimensional shape spaces: implications for morphometric applications. *Systematic Biology* **54**: 678–688.
Kubatko LS, Degnan JH. 2007. Inconsistency of phylogenetic estimates from concatenated data under coalescence. *Systematic Biology* **56**: 17–24.
Leaché AD, Rannala B. 2011. The accuracy of species tree estimation under simulation: a comparison of methods. *Systematic Biology* **60**: 126–137.
De Leonardis W, Giardina G, Zizza A. 1999. *Linaria multicaulis* (L.) Miller subsp. *humilis* (Guss.) De Leonardis, Giardina and Zizza, comb. et stat. nov., a taxon growing in Sicily. *Flora Mediterranea* **9**: 97–111.
De Leonardis W, De Santis C, Fichera G, Giardina G, Zizza A. 2003. *Linaria multicaulis* (Scrophulariaceae) in Sicily: an investigation within its subspecific and varietal ranks. *Bocconea* **16**: 585–595.
Librado P, Rozas J. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25**: 1451–1452.
Liu L. 2008. BEST: Bayesian estimation of species trees under the coalescent model. *Bioinformatics* **24**: 2542–2543.
Macior LW. 1967. Pollen-foraging behavior of *Bombus* in relation to pollination of nototribic flowers. *American Journal of Botany* **54**: 359–364.
Maddison WP. 2006. Confounding asymmetries in evolutionary diversification and character change. *Evolution* **60**: 1743–1746.
Maddison WP, Maddison DR. 2011. Mesquite: a modular system for evolutionary analysis. http://mesquiteproject.org (last accessed 24 April 2013).
Maddison WP, Midford PE, Otto SP. 2007. Estimating a binary character's effect on speciation and extinction. *Systematic Biology* **56**: 701–710.
Marazzi B, Sanderson MJ. 2010. Large-scale patterns of diversification in the widespread legume genus *Senna* and the evolutionary role of extrafloral nectaries. *Evolution* **64**: 3570–3592.
Miller MA, Pfeiffer W, Schwartz T. 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. *Proceedings of the Gateway Computing Environments Workshop (GCE) 2010*: 1–8.
Müller L. 1929. Anatomisch-biomechanische Studien an maskierten Scrophulariaceen-blüten. *Österreichische Botanische Zeitschrift* **80**: 149–161.
Newman DA, Thomson JD. 2005*a*. Effects of nectar robbing on nectar dynamics and bumblebee foraging strategies in *Linaria vulgaris* (Scrophulariaceae). *Oikos* **110**: 309–320.
Newman DA, Thomson JD. 2005*b*. Interactions among nectar robbing, floral herbivory, and ant protection in *Linaria vulgaris*. *Oikos* **110**: 497–506.
van der Niet T, Johnson SD. 2012. Phylogenetic evidence for pollinator-driven diversification of angiosperms. *Trends in Ecology & Evolution* **27**: 353–361.
Nunn CL. 2011. *The comparative approach in evolutionary anthropology and biology*. Chicago: University of Chicago Press.
Ollerton J, Alarcón R, Waser NM, *et al*. 2009. A global test of the pollination syndrome hypothesis. *Annals of Botany* **103**: 1471–1480.
Paradis E, Claude J, Strimmer K. 2004. APE: analyses of phylogenetics and evolution in R language. *Bioinformatics* **20**: 289–290.
Pérez R, Vargas P, Arroyo J. 2004. Convergent evolution of flower polymorphism in *Narcissus* (Amaryllidaceae). *New Phytologist* **161**: 235–252.
Porter ML, Crandall KA. 2003. Lost along the way: the significance of evolution in reverse. *Trends in Ecology & Evolution* **18**: 541–547.
Posada D. 2008. ModelTest: phylogenetic model averaging. *Molecular Biology and Evolution* **25**: 1253–1256.
Rabosky DL. 2010. Extinction rates should not be estimated from molecular phylogenies. *Evolution* **64**: 1816–1824.
Rabosky DL, McCune AR. 2010. Reinventing species selection with molecular phylogenies. *Trends in Ecology & Evolution* **25**: 68–74.
Rambaut A, Drummond AJ. 2007. Tracer version 1·4. http://beast.bio.ed.ac.uk/Tracer (last accessed 24 April 2013).
Robertson C. 1888. Zygomorphy and its causes. III. *Botanical Gazette* **13**: 224–230.
Rohlf FJ. 2010. tpsDig, version 2·16. http://life.bio.sunysb.edu/morph/ (last accessed 24 April 2013).
Rohlf FJ, Slice D. 1990. Extensions of the Procrustes method for the optimal superimposition of landmarks. *Systematic Zoology* **39**: 40–59.
Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572–1574.
Sáez L, Bernal M. 2009. *Linaria* Mill. In: Castroviejo S, Herrero A, Benedí C, Rico E, Güemes J. eds. *Flora Iberica. Vol. XIII*. Madrid: CSIC, 232–324.
Sánchez-Lafuente AM. 2007. Corolla herbivory, pollination success and fruit predation in complex flowers: an experimental study with *Linaria lilacina* (Scrophulariaceae). *Annals of Botany* **99**: 355–364.
Sánchez-Lafuente AM, Rodríguez-Gironés MA, Parra R. 2011. Interaction frequency and per-interaction effects as predictors of total effects in plant-pollinator mutualisms: a case study with the self-incompatible herb *Linaria lilacina*. *Oecologia* **168**: 153–165.
Sargent RD. 2004. Floral symmetry affects speciation rates in angiosperms. *Proceedings of the Royal Society Series B, Biological Sciences* **271**: 603–608.
Scherson RA, Vidal R, Sanderson MJ. 2008. Phylogeny, biogeography, and rates of diversification of New World *Astragalus* (Leguminosae) with an emphasis on South American radiations. *American Journal of Botany* **95**: 1030–1039.
Schiestl FP, Schlüter PM. 2009. Floral isolation, specialized pollination, and pollinator behavior in orchids. *Annual Review of Entomology* **54**: 425–446.
Scotland RW. 2011. What is parallelism? *Evolution & Development* **13**: 214–227.
Shipunov AB, Bateman RM. 2005. Geometric morphometrics as a tool for understanding *Dactylorhiza* (Orchidaceae) diversity in European Russia. *Biological Journal of the Linnean Society* **85**: 1–12.
Slice DE. 2001. Landmark coordinates aligned by Procrustes analysis do not lie in Kendall's shape space. *Systematic Biology* **50**: 141–149.
Smith CI, Pellmyr O, Althoff DM, Balcázar-Lara M, Leebens-Mack J, Segraves KA. 2008. Pattern and timing of diversification in *Yucca* (Agavaceae): specialized pollination does not escalate rates of diversification. *Proceedings of the Royal Society Series B, Biological Sciences* **275**: 249–258.
Smith SDW. 2010. Using phylogenetics to detect pollinator-mediated floral evolution. *New Phytologist* **188**: 354–363.
Smith SDW, Miller RE, Otto SP, FitzJohn RG, Rausher MD. 2010. The effects of flower color transitions on diversification rates in morning glories (*Ipomoea* subg. *Quamoclit*, Convolvulaceae). In: Long M, Gu H, Zhou Z. eds. *Darwin's heritage today: proceedings of the Darwin 200 Beijing International Conference*. Beijing: Higher Education Press, 202–226.
Sprengel CK. 1793. *Das entdeckte Geheimniss der Natur im Bau und in der Befruchtung der Blumen*. Berlin: F. Vieweg.
Stamatakis A. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **22**: 2688–2690.

Stanley SM. 1975. A theory of evolution above the species level. *Proceedings of the National Academy of Sciences* **72**: 646–650.

Stebbins GL. 1970. Adaptive radiation of reproductive characteristics in angiosperms, I: pollination mechanisms. *Annual Review of Ecology and Systematics* **1**: 307–326.

Stephens M, Donnelly P. 2003. A comparison of Bayesian methods for haplotype reconstruction from population genotype data. *American Journal of Human Genetics* **73**: 1162–1169.

Stephens M, Smith NJ, Donnelly P. 2001. A new statistical method for haplotype reconstruction from population data. *American Journal of Human Genetics* **68**: 978–989.

Stout JC, Allen JA, Goulson D. 2000. Nectar robbing, forager efficiency and seed set: bumblebees foraging on the self incompatible plant *Linaria vulgaris* (Scrophulariaceae). *Acta Oecologica* **21**: 277–283.

Suc JP. 1984. Origin and evolution of the Mediterranean vegetation and climate in Europe. *Nature* **307**: 429–432.

Sutton DA. 1980. A new section of *Linaria* (Scrophulariaceae: Antirrhineae). *Botanical Journal of the Linnean Society* **81**: 169–184.

Sutton DA. 1988. *A revision of the tribe Antirrhineae*. Oxford: Oxford University Press.

Tan K, Iatrou G. 2001. *Endemic plants of Greece: the Peloponnese*. Copenhagen: Gad.

Tripp EA, Manos PS. 2008. Is floral specialization an evolutionary dead-end? pollination system transitions in *Ruellia* (Acanthaceae). *Evolution* **62**: 1712–1737.

Valdés B. 1970. Taxonomía experimental del género *Linaria* IV. Reproducción sexual: autogamia y polinización intraespecífica. *Boletín de la Real Sociedad Española de Historia Natural. Sección Biológica* **68**: 79–89.

Valente LM, Savolainen V, Vargas P. 2010*a*. Unparalleled rates of species diversification in Europe. *Proceedings of the Royal Society Series B, Biological Sciences* **277**: 1489–1496.

Valente LM, Reeves G, Schnitzler J, *et al*. 2010*b*. Diversification of the African genus *Protea* (Proteaceae) in the Cape biodiversity hotspot and beyond: equal rates in different biomes. *Evolution* **64**: 745–760.

Valente LM, Manning JC, Goldblatt P, Vargas P. 2012. Did pollination shifts drive diversification in Southern African *Gladiolus*? Evaluating the model of pollinator-driven speciation. *American Naturalist* **180**: 83–98.

Vargas P, Rosselló JA, Oyama R, Güemes J. 2004. Molecular evidence for naturalness of genera in the tribe Antirrhineae (Scrophulariaceae) and three independent evolutionary lineages from the New World and the Old. *Plant Systematics and Evolution* **249**: 151–172.

Vargas P, Ornosa C, Ortiz-Sanchez FJ, Arroyo J. 2010. Is the occluded corolla of *Antirrhinum* bee-specialized? *Journal of Natural History* **44**: 1427–1443.

Viano J. 1969. Note sur le genre *Linaria* en Méditerranée occidentale. Le groupe *Linaria bipartita* (Vent.) Willd. *Naturalia Monspeliensia, Série Botanique* **20**: 219–240.

Viano J. 1978*a*. Les linaires à graines aptères du bassin méditerranéen occidental. 1. *Linaria* sect. *Versicolores*. *Candollea* **33**: 33–88.

Viano J. 1978*b*. Les linaires à graines aptères du bassin méditerranéen occidental. 2. *Linaria* sect. *Elegantes*, *Bipunctatae*, *Diffusae*, *Speciosae*, *Repentes*. *Candollea* **33**: 209–267.

Wake DB. 1991. Homoplasy: the result of natural selection, or evidence of design limitations? *American Naturalist* **138**: 543–567.

Wake DB, Wake MH, Specht CD. 2011. Homoplasy: from detecting pattern to determining process and mechanism of evolution. *Science* **331**: 1032–1035.

Waser NM, Chittka L, Price MV, Williams NM, Ollerton J. 1996. Generalization in pollination systems, and why it matters. *Ecology* **77**: 1043–1060.

Wertheim JO, Sanderson MJ. 2011. Estimating diversification rates: how useful are divergence times? *Evolution* **65**: 309–320.

Whittall JB, Hodges SA. 2007. Pollinator shifts drive increasingly long nectar spurs in columbine flowers. *Nature* **447**: 706–709.

Yang CF, Gituru RW, Guo YH. 2007. Reproductive isolation of two sympatric louseworts, *Pedicularis rhinanthoides* and *Pedicularis longiflora* (Orobanchaceae): how does the same pollinator type avoid interspecific pollen transfer? *Biological Journal of the Linnean Society* **90**: 37–48.

Yu Y, Than C, Degnan JH, Nakhleh L. 2011. Coalescent histories on phylogenetic networks and detection of hybridization despite incomplete lineage sorting. *Systematic Biology* **60**: 138–149.

Yu Y, Degnan JH, Nakhleh L. 2012. The probability of a gene tree topology within a phylogenetic network with applications to hybridization detection. *PLoS Genetics* **8**: e1002660.

Zelditch ML, Swiderski DL, Sheets HD, Fink WL. 2004. *Geometric morphometrics for biologists: a primer*. San Diego: Elsevier Academic.

Appendix 3

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EN BÚSQUEDA DE ÁREAS DE DIVERSIDAD GENÉTICA EN SIERRA NEVADA: ANÁLISIS DE PLANTAS Y ABEJAS

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RESUMEN

El estudio de la biodiversidad por medio de la genética no solo sirve para obtener un conocimiento más preciso de la diversidad críptica según áreas geográficas sino también para poder señalar las poblaciones mejor preparadas para afrontar cambios climáticos y evolutivos. En el presente estudio se exponen los principales resultados de la localización de áreas ricas en biodiversidad genética dentro del Parque Nacional de Sierra Nevada, sobre la base del número de genotipos (haplotipos de los genomas del plasto y la mitocondria) y linajes de haplotipos. Las especies finalmente seleccionadas fueron tres plantas endémicas (*Chaenorhinum glareosum*, *Linaria glacialis* y *Linaria nevadensis*) y una abeja (*Apis mellifera*), que es un "superpolinizador" de la flora de Sierra Nevada. Además se elaboró un catálogo de las especies de abejas conocidas en la alta montaña nevadense. Finalmente detectamos dos áreas (macizos de El Caballo y Puntal Vacares-Pico Cuervo) que albergan más de la mitad de la variación genética de, al menos, dos de las cuatro especies analizadas. Este novedoso enfoque de localización del patrimonio genético de Sierra Nevada sirve además para priorizar áreas de conservación máxima (microrreservas) que se podrían crear dentro de cualquier parque nacional.

Palabras clave: abejas, *Apis*, *Antirrhinum*, biodiversidad, *Chaenorhinum*, *Linaria*, mitocondria, plasto, secuencias.

SUMMARY

The assessment of biodiversity based on genetics not only provides a precise knowledge of cryptic diversity from geographical areas, but also helps pinpoint the populations better fitted to cope with climatic and evolutionary changes. The present study shows the main results after searching for rich areas of biodiversity within the National Park of Sierra Nevada using number of genotypes (haplotypes from the plastid and mitochondria genomes) and lineages of haplotypes. Three endemic plant species (*Chaenorhinum glareosum*, *Linaria glacialis* and *Linaria nevadensis*) and a bee (*Apis mellifera*), which is a "superpollinator" of the flora of Sierra Nevada, were eventually chosen. In addition, we built up a check-

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list of species of bees occurring in high altitudes. Two main areas (El Caballo and Puntal Vacares-Pico Cuervo massifs) harbour more than half of the genetic variation observed based, at least, on two of the four species analysed. This new approach to locate genetic diversity across Sierra Nevada help prioritize areas of special conservation that are suggested to be created in any national park.

Key words: *Apis*, *Antirrhinum*, bees, biodiversity, *Chaenorhinum*, *Linaria*, mitochondria, plast, sequences.

INTRODUCCIÓN

Cualquier profesional en biología que asciende a las cotas superiores del Parque Nacional de Sierra Nevada (PNSN) se da cuenta de que desaparecen los bosques, e incluso los arbustos, y de que la vegetación pasa a estar compuesta por un bajo número de especies herbáceas, que incluso aparecen en menor número que en cotas más bajas (BLANCA *et al*., 2009). Lo mismo ocurre con la diversidad en el número de especies de animales. En estas cotas elevadas también el número de hábitats se reduce, todo ello como consecuencia del efecto del descenso de las temperaturas en la biodiversidad. Si exceptuamos las zonas ecotónicas, donde las pedreras y roquedos se encuentran con sistemas lacustres y fluviales, es difícil distinguir zonas ricas y pobres, y por tanto predecir en qué áreas de alta montaña se encuentra una mayor diversidad.

El concepto de biodiversidad debe reflejar la variedad de seres vivos de un área y los patrones bióticos que la conforman. Una primera y rápida evaluación de la biodiversidad de un área consiste en contar el número de especies. Pero la biodiversidad es mucho más compleja que el número de especies (diversidad taxonómica) y su abundancia, pues también debe incluir una diversidad más críptica (GIBSON & DWORKIN, 2004). Nos referimos a la diversidad genética, que no solo sirve para obtener un nivel más preciso de diversidad por áreas sino también para señalar las poblaciones mejor preparadas para afrontar cambios climáticos y evolutivos. A mayor diversidad genética mayor probabilidad de sobrevivir a cambios en el ambiente. Por tanto, las poblaciones con poca diversidad genética tienen mayor riesgo y una respuesta probabilísticamente más inadecuada ante los impredecibles cambios ambientales.

En este estudio se eligió uno de los grupos de plantas que tienen una asociación más estrecha con la polinización por abejas. Las especies de bocas de dragón (*Antirrhinum*) y parientes próximos (tribu Antirrhineae, Plantaginaceae) tienen una corola ocluida (flor personada) constituida por dos partes (labios): una de tres pétalos inferiores trasformados en una estructura (paladar) que se cierra con el labio superior de dos pétalos. Las abejas tienen la habilidad y fuerza para abrir esta corola hermética para otros polinizadores, recoger y diseminar el polen. Para obtener resultados que caractericen mejor Sierra Nevada, elegimos las especies endémicas de esta tribu taxonómica de manera que todos los procesos de diferenciación se hayan producido en el PNSN y aledaños. Sin embargo, no hay consenso taxonómico para todas ellas, especialmente para *Antirrhinum rupestre* Boiss. & Reut., *Linaria nevadensis* (Boiss.) Boiss. & Reut., *Linaria verticillata* Boiss. y *Linaria saturejoides* Boiss. subsp. *angustifolia* (Wilmott) L. Sáez & M. B. Crespo. Por ello, empleamos métodos filogenéticos, basados en las mismas regiones de DNA que las usadas en el estudio de diversidad genética, para determinar grupos monofiléticos dentro de los géneros *Antirrhinum*, *Chaenorhinum* y *Linaria*. Así pudimos tomar decisiones propias sobre la sistemática de la especie y selección final de las mismas sobre la base de patrones evolutivos. En concreto, las especies elegidas nos sirven para valorar los siguientes aspectos en la conservación y gestión del PNSN: (1) utilidad de las técnicas de *barcoding* para detectar diversidad genética

en extensiones geográficamente pequeñas; (2) estructuración de la diversidad genética en áreas de montaña; (3) localización de lugares y áreas de mayor y menor riqueza genética; (4) priorización en la conservación de poblaciones y especies indicadoras; y (5) priorización en la conservación de poblaciones que favorecen la cohesión entre organismos (interacciones planta-animal).

El objetivo principal de este estudio ha sido obtener resultados fundamentales sobre la biodiversidad del PNSN por medio del listado del número de insectos polinizadores (abejas) en un gradiente que incluye los picos, collados y laderas donde viven ciertas especies de angiospermas (especies de boca de dragón, Antirrhineae, Plantaginaceae) con las que interaccionan (diversidad funcional). El siguiente paso en la búsqueda de biodiversidad fue analizar la diversidad de genes indicadores (diversidad genética) tanto de las plantas como de las abejas polinizadoras. Finalmente los objetivos reevaluados fueron: (1) describir la riqueza de insectos (abejas) polinizadores en cuatro especies de Antirrhineae (*Antirrhinum rupestre*, *Chaenorhinum glareosum*, *Linaria glacialis* y *Linaria nevadensis*); (2) analizar la diversidad genética de las cuatro especies de Antirrhineae; (3) analizar la diversidad genética de las abejas polinizadoras; y (4) localizar las áreas de Sierra Nevada con mayor diversidad genética de plantas y abejas para proponer lugares específicos para su conservación (microrreservas).

MATERIAL Y MÉTODOS

Muestreo

Durante los veranos de 2009-2012 se visitaron los lugares donde estaban citadas poblaciones de las cuatro especies evaluadas de angiospermas: *Antirrhinum rupestre* (piso de vegetación supramediterráneo), *Linaria nevadensis* (piso de vegetación oromediterráneo), *Linaria glacialis* (piso de vegetación crioromediterráneo) y *Chaenorhinum glareosum* (piso de vegetación crioromediterráneo) (Lámina 1). Es decir, se muestreó una franja altitudinal desde 1.000 m hasta las cimas de los macizos por encima de 2.700 m (piso crioromediterráneo) (Anexo 1). Otras especies de Antirrhineae fueron finalmente descartadas (véanse criterios más adelante).

Además en estas campañas de toma de material y datos se realizaron censos de polinización de las cuatro plantas sujetos de estudio cuando las condiciones son ideales para la observación de abejas (sin viento, sin lluvia, temperatura > 20 ºC). Se muestreó un total de 19 localidades para la recolección de tejido vegetal y la observación de polinizadores (véase Anexo 1).

Fauna polinizadora de Sierra Nevada

Las dudas acerca de la validez taxonómica de algunas especies de Antirrhineae (véanse más adelante) y el hecho de que el número de especies de abejas visitando las especies de alta montaña de Antirrhineae haya sido muy bajo (una sola especie de abeja en *Linaria nevadensis*), nos condujo a cambiar de estrategia respecto a lo inicialmente proyectado. Asimismo el número de especies de abejas visitando otras especies de flora por encima de 3.000 m fue muy escaso durante los años de estudio. Dado que

Anexo 1. Códigos empleados para numerar las localidades y poblaciones muestreadas en el presente estudio (Figuras 1-5).

L. glacialis: 1, Veleta NW; 2, Veleta SW; 3, Mulhacén; 4, Cerro de los Machos; 5, Tozal del Cartujo; 6, El Caballo; 7, Puntal de los Cuartos; 8, Cerro San Juan; 9, Pico del cuervo/ Puntal de Vacares; 10, Pico del Globo. **Linaria nevadensis**: 1, Veleta NW; 3, Mulhacén; 5, Tozal del Cartujo; 6, El Caballo; 11, Pico del Cuervo; 12, Puntal de Vacares; 13, Puntal de Trévelez; 18, Laguna Hondera; 19, Acucaderos; 20, Hoya de la Mora; 21, Morrón Sanjuanero; 22, Pradollano; 23, Puntal de los Cuartos; 24, San Juan. **Chaenorhinum glareosum**: 1, Veleta; 3, Mulhacén; 5, Tozal del Cartujo; 6, El Caballo; 8, Cerro San Juan; 11, Pico del Cuervo; 12, Puntal de Vacares; 13, Puntal de Trévelez; 14, Portal; 15, Culo Perro; 16, Pico Mulhacén. **Antirrhinum rupestre** (A. hispanicum): 25, Barranco de las viboras; 26, Pradollano; 27, Abrucena; 28, Trevenque; 29, Güejar-Sierra. **Apis mellifera iberiensis**: 1, Veleta; 3, Mulhacén; 6, El Caballo; 9, Pico del cuervo/ Puntal de Vacares; 17, Tajos del Goterón; 18, Laguna Hondera.

algunas especies de plantas dejaban de ser incluidas en el estudio por falta de lógica evolutiva y validez sistemática, incluimos dos nuevos objetivos: (i) compilar un catálogo de las abejas observadas visitando la flora de Sierra Nevada, y (ii) realizar los estudios de diversidad genética (secuenciación) en las tres especies de abejas más frecuentes en alta montaña. Consideramos que este reenfoque podría ayudar a una mejor gestión de la fauna polinizadora del PNSN.

Estudios piloto de las plantas endémicas de Sierra Nevada

Como quiera que la divergencia genética en animales y plantas suele estar asociada a la distancia geográfica, inicialmente se realizaron estudios piloto de diversidad genética basados en un muestreo geográficamente representativo. Así, se seleccionaron cuatro poblaciones (una muestra por población) geográficamente alejadas entre sí para comprobar dos aspectos fundamentales: (i) si las especies forman grupos monofiléticos, i. e. si tienen un solo origen y la diversidad genética se ha generado únicamente en Sierra Nevada, y (ii) si las regiones de DNA elegidas a partir de técnicas de *barcoding* (véase más abajo) son suficientemente variables. Posteriormente se amplió el muestro a un número mayor de individuos y poblaciones que representó la distribución de las especies.

Técnicas de DNA *barcoding* para localizar áreas de diversidad

El DNA *barcoding* es una herramienta taxonómica en la que se usa la mínima caracterización de DNA que permita identificar una determinada especie y que a la vez la separe de las más próximas. Además, son regiones del genoma que han resultado ser de gran valor evolutivo, ya que proporcionan una señal filogenética de confianza en numerosas familias y géneros de angiospermas. En este estudio se han empleado varias regiones de DNA universalmente probadas en plantas y animales, de manera que definan cada especie sujeto de estudio y que además proporcionen suficiente diversidad genética para caracterizar sus poblaciones. Los estudios piloto de las especies de Antirrhineae incluyeron 10-20 regiones del DNA plastidial. Finalmente se seleccionaron y secuenciaron diferentes regiones espaciadoras dependiendo de la variabilidad encontrada en cada especie: *rpl*32-*trn*L^{UAG}, *trn*S-*trn*G, *trn*Q-*rps*16 y *rps*16-*trn*K (VARGAS *et al*., 2009; FERNÁNDEZ-MAZUECOS & VARGAS, 2011; BLANCO-PASTOR *et al*., 2012). Para el caso de las abejas, las regiones de DNA están mejor establecidas debido a su comprobada utilidad, por lo que ensayamos directamente una de las más variables, i. e. el espaciador mitocondrial COI-COII (CORNUET *et al*., 1991; FRANCK *et al*., 1998; RORTAIS *et al*. 2011).

Análisis genéticos

Para estimar la diversidad genética de plantas y abejas, se obtuvieron resultados según la diversidad de genotipos y de linajes de estos genotipos. Las regiones utilizadas pertenecen a los genomas organulares (plasto y mitocondria), que están formados por una única molécula no recombinante, por lo que el estudio se basó en el número de genotipos haploides (haplotipos). Para que el estudio dispusiera de diversidad genética sustancial, la comparación entre secuencias de las regiones estudiadas debería mostrar varios cambios nucleotídicos (no se emplean inserciones/delecciones debido a su conocida homoplasia) dentro de cada especie. Es decir, se analizaron secuencias de decenas de individuos (una por individuo) que difirieran (y no) en cambios nucleotídicos a lo largo de la región secuenciada. Una vez obtenidos un buen número de haplotipos por especie (> 10) se analizó su distribución geográfica en Sierra Nevada. Para valorar la repartición de la diversidad genética de cada especie en Sierra Nevada, se analizó el número de haplotipos y el número de linajes de haplotipos (relaciones de haplotipos por parentesco) de cada población.

RESULTADOS

Polinizadores de las especies de Antirrhineae

Un total de 12 especies de abejas fueron encontradas accediendo a las flores de las especies de Antirrhineae seleccionadas (Tabla 1). Información filogenética de la tribu Antirrhineae procedente de análisis que estamos llevando a cabo en paralelo en la tribu Antirrhineae desaconsejó, al poco tiempo de empezar el presente estudio, incluir ciertas subespecies subendémicas, que además tenían escaso valor taxonómico, debido a que estos táxones no forman grupos monofiléticos. Por ello realizamos censos de polinizadores en *Antirrhinum rupestre*, *Chaenorhinum glareosum*, *Linaria glacialis* y *Linaria nevadensis*. Como se preveía inicialmente (VARGAS *et al*., 2010), prácticamente solo especies de abejas entraron en las flores de las especies de Antirrhineae (Tabla 1). Además, una polilla (*Macroglossum stellatarum*, Sphingidae), fue observada en dos especies de Antirrhineae.

Antirrhinum rupestre Boiss. & Reut

Se realizaron censos de polinizadores de esta especie en tres poblaciones empleadas para el estudio de diversidad genética (Barranco de las Víboras, Trevenque y Güéjar-Sierra). En la población de Barranco de las Víboras el censo se realizó sobre un parche de 50 flores (promedio) durante 660 minutos. Las abejas que visitaron legítimamente las flores fueron: *Anthophora crassipes* (4 visitas), *Lasioglossum interruptum* (2), *Chalicodoma pyrenaica* (1), *Bombus terrestris* (1) y *Anthophora mucida* (1). También se observó la visita del lepidóptero *Macroglossum stellatarum* (1). En la población del Trevenque el censo se realizó sobre un parche de cerca de 40 flores (promedio) durante 465 minutos. Las abejas que visitaron legítimamente las flores fueron: *Anthophora crassipes* (18 visitas), *Lasioglossum buccale* (12), *Chalicodoma pyrenaica* (11) y *Rhodanthidium sticticum* (5). En la población de Güéjar-Sierra el censo se realizó sobre un parche de 35 flores (promedio) durante 310 minutos. Las abejas que visitaron legítimamente las flores fue-

Especies de plantas	nº. minutos	Especies de abejas	Especies de Lepidópteros
Antirrhinum rupestre Boiss. & Reut.	1435	*Anthidium manicatum* (Linnaeus, 1758) *Anthidium oblongatum* (Illiger, 1806) *Anthophora crassipes Lepeletier,* 1841 *Anthophora mucida Gribodo*, 1873 *Bombus terrestris* (Linnaeus, 1758) *Chalicodoma pyrenaica Lepeletier*, 1841 *Lasioglossum interruptum* (Panzer, 1798) *Lasioglossum buccale* (Pérez,1903) *Rhodanthidium sticticum* (Fabricius, 1787) *Osmia sp. Panzer*, 1806	*Macroglossum stellatarum* Linnaeaus, 1758
Chaenorhinum glareosum (Boiss.) Willk	c. 1000	*Bombus terrestris* (Linnaeus, 1758) *Apis mellifera* Linnaeus, 1758	*Macroglossum stellatarum* Linnaeus,1758
Linaria glacialis Boiss.	c. 2000	–	–
Linaria nevadensis Boiss. & Reuter	1360	*Chalicodoma parietina* (Geoffroy, 1785) *Bombus terrestris* (Linnaeus, 1758)	–

Tabla 1. Número de visitas resultantes de los censos de polinizadores de las especies de Antirrhineae.

Table 1. Number of visits observed on Antirrhineae species in the pollination censuses.

ron: *Chalicodoma pyrenaica* (30 visitas), *Bombus terrestris* (12), *Anthidium manicatum* (4), *Lasioglossum buccale* (3), *Osmia* sp. (2) y *Anthidium oblongatum* (2).

Chaenorhinum glareosum (Boiss.) Willk

Se realizaron censos de esta especie en tres poblaciones (La Carihuela, Veleta y Pico Veleta- Pico del Cuervo). En la población de La Carihuela se realizó un censo durante 90 minutos. La única abeja que visitó legítimamente las flores fue *Bombus terrestris* (1 visita). También se observó la visita del lepidóptero *Macroglossum stellatarum* (6 visitas). Fuera del censo se observó *Apis mellifera* en una flor. En la población del Veleta se realizó un censo durante 90 minutos. La abeja que visitó legítimamente las flores fue *Bombus terrestris* (3 visitas). También se observó la visita del lepidóptero *Macroglossum stellatarum* en otras de sus flores fuera del censo. En el transecto del Pico Veleta-Pico del Cuervo (4 días) solo se observó *Macroglossum stellatarum* accediendo a las flores de esta planta.

Linaria glacialis Boiss

Esta especie no presenta ni un número elevado de individuos por m^2 (normalmente 1-8) ni de flores por individuo (normalmente 1-4 en flor a la vez), por lo que ningún parche proporcionó un número elevado de flores. Se realizaron censos de esta especie en cinco poblaciones (La Carihuela, Lavaderos de la Reina, Veleta, Cerro de los Machos, Tozal del Cartujo). En la población de La Carihuela se realizó un censo durante 90 minutos pero no se observó ninguna visita. El mismo resultado se obtuvo en un censo de 90 minutos en los Lavaderos de la Reina, en otro censo de 360 minutos en el Veleta, en otro censo de 240 minutos en el Cerro de los Machos y en un transecto de 60 minutos en el Tozal del Cartujo. Otros transectos con resultados negativos se realizaron en el Puntal de Vacares (120 minutos) y el Mulhacén (240 minutos).

Linaria nevadensis (Boiss.) Boiss & Reut

Se realizaron censos y transectos para esta especie en cinco poblaciones (Hoya de la Mora, Laguna Hondera, Tajos del Goterón, Laguna de Vacares y Borreguiles). En la población de la Hoya de la Mora se realizó un censo sobre un parche de 70 flores (promedio) durante 270 minutos. Las abejas que visitaron legítimamente las flores fueron: *Chalicodoma parietina* (9 visitas) y *Bombus terrestris* (2). En la población de la Laguna Hondera se realizó un transecto durante 60 minutos. La única abeja que visitó legítimamente las flores fue *Chalicodoma parietina* (5 visitas). En la población de los Acucaderos se realizó un transecto durante 600 minutos. La única abeja que visitó legítimamente las flores fue *Chalicodoma parietina* (12 visitas). En la población de la Laguna de Vacares se realizó un transecto durante 30 minutos. La única abeja que visitó legítimamente las flores fue *Chalicodoma parietina* (1 visita). En la población de los Borreguiles se realizó un censo durante 460 minutos. Las única abeja que visitó legítimamente las flores fue *Chalicodoma parietina* (170 visitas). Otros transectos con resultados negativos se realizaron en el Caballo (2 horas) y Pico del Cuervo (7 horas). En esta última localidad fue observada *Chalicodoma parietina* en flores de otras especies de angiospermas.

Especies de abejas en zonas altas de Sierra Nevada

En la Tabla 2 se muestran las abejas observadas en cotas medias y altas de Sierra Nevada. Además de las especies capturadas por nosotros en esa tabla se incluyen aquellas recogidas en la literatura. Aunque se hayan observado un total de 44 especies de abejas en zonas altas, dos (*Bombus terrestris* y *Apis mellifera*) han resultado ser "superpolinizadores", es decir polinizadores de la mayor parte de la flora alpina de Sierra Nevada. Una más (*Chalicodoma parietina*) se ve más esporádicamente, si bien es el polinizador principal de una de las especies de Antirrhineae (*Linaria nevadensis*). A continuación resumimos características básicas de esta especie de abeja y de los dos "superpolinizadores" de la flora de Sierra Nevada (Lámina 1).

Especies de abejas	Localidad, altitud y referencias	Número de individuos recolectados
Andrena afrensis Warncke, 1967	2.160 m (Gómez & Zamora 1999)	–
Andrena carbonaria (Fabricius, 1781)	2.160 m (Gómez & Zamora 1999)	–
Andrena nigroaenea (Kirby, 1802)	2.160 m (Gómez & Zamora 1999)	–
Andrena niveata Friese, 1887	2.160 - 3.130 m (Gómez & Zamora 1999)	–
Andrena ovatula (Kirby, 1802)	2.160 - 2.550 m (Gómez & Zamora 1999)	–
Andrena similis Smith, 1849	2.160 - 3.130 m (Gómez & Zamora 1999)	–
Anthidium manicatum (Linnaeus, 1758)	Güéjar-Sierra y Barranco de las Víboras, 1100-1500 m (JLB) (este trabajo)	1
Anthidium montanum Morawitz, 1864	Citado para "Sierra Nevada" (Ebmer 2003)	–
Anthidium oblongatum (Illiger, 1806)	Güéjar-Sierra, c. 1500 m (JLB) (este trabajo)	1
Anthophora crassipes Lepeletier, 1841	Barranco de las Víboras y Trevenque, c. 1500 m (CO, JLB y PV) (este trabajo)	18
Anthophora furcata (Panzer, 1798)	Pradollano, 2.040 m (Ortiz-Sánchez *et al.* en prensa)	–
Anthophora mucida Gribodo, 1873	Barranco de las Víboras, 1500 m (PV) (este trabajo)	1
***Apis mellifera iberiensis* Engel, 1999**	**2.500–3.400 m (CO, JLB y PV) (este trabajo)**	**56**
Bombus maxillosus Klug, 1817	Puerto de la Ragua, 2.270 m (PV) (este trabajo)	2
Bombus pascuorum bofilli Vogt 1911	Caballo, 2.320 m (76PV11) (este trabajo)	2
Bombus pratorum (Linnaeus, 1761)	Veleta, 3.300 m (66PV11) (este trabajo)	1
Bombus reinigiellus (Rasmont, 1983)	1.900–3.255 m (Ortiz-Sánchez *et al.* en prensa)	–
Bombus ruderatus rondensis Castro, 1991	800–1.650 m (Ortiz-Sánchez *et al.* en prensa)	–
Bombus ruderatus ruderatus (Fabricius, 1775)	Caballo 2.320 m (75PV11a y b), 2.800 m (78PV11a) (este trabajo)	3
***Bombus terrestris lusitanicus* Krüger, 1956**	**Hasta 3.400 m (CO, JLB y PV) (este trabajo)**	**32**
***Bombus terrestris terrestris* (Linnaeus, 1758)**	**Normalmente hasta 2.200 m (CO, JLB y PV) (este trabajo)**	**6**
Ceratina cucurbitina (Rossi, 1792)	Güejar-Sierra, 1.200 m (JLB) (este trabajo)	1
Chalicodoma albonotata (Radoszkowski, 1886)	Pradollano, 2.200 m (JLB) (este trabajo)	2
Chalicodoma parietina (Geoffroy, 1785)	2.100-3.400 m (CO, JLB y PV) (este trabajo; véase también Ortiz-Sánchez *et al.*, en prensa)	32
Chalicodoma pyrenaica Lepeletier, 1841	Barranco de las Víboras, Güéjar-Sierra y Trevenque, 1.100-1800 m (CO, JLB y PV) (este trabajo)	14
Colletes carinatus Radoszkowski, 1891	Borreguiles del río, Monachil, 2.700 m (Ortiz-Sánchez, Ornosa *et al.* en prensa)	–
Colletes floralis Eversmann, 1852	2.800–2.900 m (Ortiz-Sánchez *et al.* en prensa)	–
Colletes nigricans Gistel, 1857	2.550 m (Gómez & Zamora 1999)	–
Colletes schmidi Noskiewicz, 1962	1.200–3.000 m (Ortiz-Sánchez *et al.* en prensa)	–
Dasypoda albimana Pérez, 1905	Fuente de Don Manuel, Monachil, 2.100 m (Ortiz-Sánchez, Ornosa *et al.* en prensa)	–
Dasypoda morotei (Quilis, 1928)	Veleta, 3.200 m (CO) (este trabajo)	1
Dufourea paradoxa nivalis Ebmer, 1989	2.700–3.100 m (Ortiz-Sánchez *et al.* en prensa)	-
Flavipanurgus granadensis (Warncke, 1987)	1.300–1.430 m (Ortiz-Sánchez *et al.* en prensa)	-

(continúa)

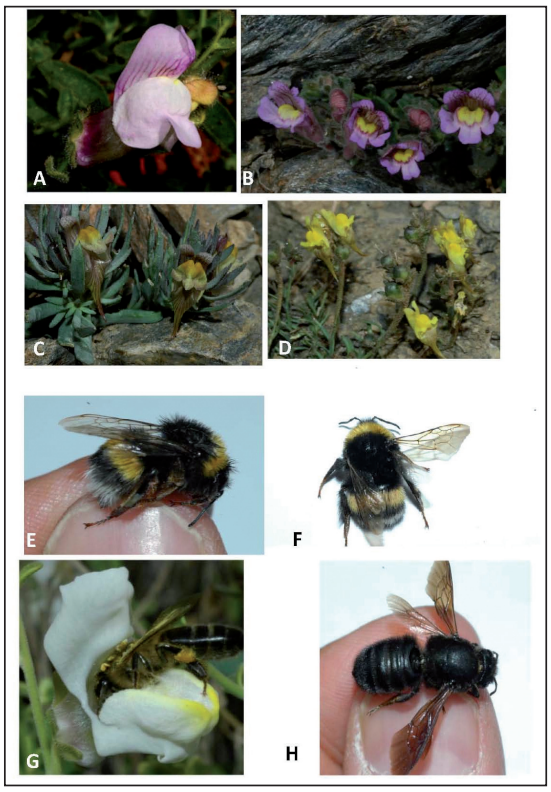
(continuación)

Especies de abejas	Localidad, altitud y referencias	Número de individuos recolectados
Halictus nivalis Ebmer, 1985	2.500–3.100 m (Ortiz-Sánchez *et al.* en prensa)	-
Hoplitis claviventris columba (Warncke, 1991)	Puerto de la Ragua, 1.700 m (Ortiz-Sánchez *et al.* en prensa)	-
Hoplitis mitis granadae Tkalc, 1984	2.500–2.600 m (Ortiz-Sánchez *et al.* en prensa)	-
Hoplitis ochraceicornis (Ferton, 1902)	Citado para "Sierra Nevada", 1.400 m (Warncke, 1992)	-
Hoplitis praestans (Morawitz, 1893)	Collado de Las Sabinas, 2.170 m (JLB) (este trabajo)	2
Hoplitis ravouxi (Pérez, 1902)	Pico Veleta, 2.850 m (Ortiz-Sánchez *et al.* en prensa)	-
Hylaeus euryscapus Förster, 1871	2.550 m (Gómez & Zamora 1999)	-
Lasioglossum buccale (Pérez, 1903)	Güéjar-Sierra y Trevenque, 1.100-500 m (CO, JLB y PV) (este trabajo)	6
Lasioglossum interruptum (Panzer, 1798)	Barranco de las Víboras, 1500 m (PV) (este trabajo)	1
Lasioglossum leucozonium elysium Ebmer, 1979	Puerto de la Ragua, 2.000 m (Ortiz-Sánchez *et al.* en prensa)	-
Lasioglossum malachurum (Kirby, 1802)	2.550 m (Gómez & Zamora 1999)	-
Lasioglossum mediterraneum (Blüthgen, 1926)	2.160 - 3.130 m (Gómez & Zamora 1999)	-
Lasioglossum pauperatum (Brullé, 1832)	Veleta, 2.800 m (82JLB09) (este trabajo)	1
Lasioglossum soror (Saudners, 1901)	2.160 m (Gómez & Zamora 1999)	-
Megachile analis Nylander, 1852	2.500–3.100 m (Ortiz-Sánchez *et al.* en prensa)	-
Megachile lagopoda (Linnaeus, 1761)	Más de 2.000 m (Ortiz-Sánchez *et al.* en prensa)	-
Megachile maritima (Kirby, 1802)	Distintas localidades, orófilo (Ortiz-Sánchez *et al.* en prensa)	-
Melitta iberica Warncke, 1973	Hasta 2.400 m (Ortiz-Sánchez *et al.* en prensa)	-
Osmia cyanoxantha Pérez, 1879	Citado para "Sierra Nevada", 2.250 m (Zanden 1991)	-
Osmia emarginata Lepeletier, 1841	carretera al Veleta, Parking Los Peñones, 2.100 m (JLB) (este trabajo)	1
Osmia gallarum Spinola, 1808	Collado de Las Sabinas, 2.170 m (JLB) (este trabajo)	1
Osmia labialis Pérez, 1879	Barranco de San Juan, 2.600 m (Ortiz-Sánchez *et al.* en prensa)	-
Osmia parietina Curtis, 1928	Collado de Las Sabinas, 2.170 m (JLB) (este trabajo)	1
Osmia saxicola Ducke, 1899	Veleta, 2.880 m (Ortiz-Sánchez *et al.* en prensa)	-
Osmia viridana Morawitz, 1874	Citado para "Sierra Nevada", 2.250 m (Warncke 1991)	-
Protosmia stigmatica (Pérez, 1895)	Puerto de la Ragua, 1.800 m (Ortiz-Sánchez *et al.* en prensa)	-
Psithyrus vestalis obenbergeri May, 1944	Hasta 2.600 m (Ortiz-Sánchez *et al.* en prensa)	-
Rhodanthidium sticticum (Fabricius, 1787)	Trevenque, c. 1500 m (JLB) (este trabajo)	1
Stelis breviuscula Nylander, 1848	Puerto de la Ragua, 2.000 m (Ortiz-Sánchez *et al.* en prensa)	-
Stelis minuta Lepeletier & Serville, 1825	Cita antigua "VII-16-21" de "Sierra Nevada" (Ornosa *et al.* 2009)	-
Stelis punctulatissima (Kirby, 1802)	Pradollano, 2.040 m (Ortiz-Sánchez *et al.* en prensa)	-
Xylocopa violacea (Linnaeus, 1758)	Veleta, 2.708 m (CO) (este trabajo)	2

Tabla 2. Catálogo de las abejas de Sierra Nevada. En negrita los polinizadores principales ("superpolinizadores") de la flora de alta montaña de Sierra Nevada. Se indican las especies por orden alfabético, las altitudes y códigos de captura, número de individuos y referencias bibliográficas. Abreviaturas de recolectores: CO, Concepción Ornosa; JLB, José Luis Blanco-Pastor; PV, Pablo Vargas.

Table 2. Ckeck-list of bee species from Sierra Nevada. In black main pollinators for the alpine flora of Sierra Nevada. Species names, locations, altitude, collecting codes, number of individuals and bibliographic references are indicated. Collector abbreviations: CO, Concepción Ornosa; JLB, José Luis Blanco-Pastor; PV, Pablo Vargas.

Lámina 1. Especies clave del presente estudio. A, *Antirrhinum rupestre*; B, *Chaenorhinum glareosum*; C, *Linaria glacialis*; D, *Linaria nevadensis*; E, *Bombus terrestris lusitanicus*; F, *Bombus terrestris terrestris*, G. *Apis mellifera iberiensis*; H. *Chalicodoma parietina*. Fotografías Pablo Vargas y José Luís Blanco-Pastor.



Bombus terrestris (Linnaeus, 1758)

Abejorro paleártico de hábitos sociales, nidificación subterránea y especie polinizadora por excelencia de montañas y zonas frías, pero también distribuida en zonas bajas. Ha sido introducida en gran parte del planeta, incluidos territorios tan lejanos como los americanos del norte (EEUU) y del sur (Brasil y Chile) o Japón, Tasmania y Nueva Zelanda. En Sierra Nevada están representadas las dos subespecies ibéricas: *Bombus terrestris terrestris* y *Bombus terrestris lusitanicus*, si bien la subespecie *lusitanicus* es predominante (32 de 38 ejemplares capturados). No es infrecuente, de cualquier modo, hallar formas híbridas. *Bombus terrestris lusitanicus* presenta la clásica coloración a bandas amarillas, negras y blanca en que el color negro tiende a ser sustituido por un tono pardo herrumbroso, muy patente ventralmente y en las patas (Lámina 1). Esta subespecie se reparte por la Península Ibérica y las islas Baleares, Madeira y penetra en el sur de Francia (ORNOSA & ORTIZ-SÁNCHEZ, 2004). En cuanto a su distribución altitudinal, lo más frecuente es hallarla por debajo de 3.000 m; sin embargo en Sierra Nevada la hemos capturado hasta los 3.400 m. *Bombus terrestris terrestris* se caracteriza por una coloración a bandas amarillas, negras y blanca, sin coloración parda herrumbrosa. Su dispersión natural es paleártica occidental, penetrando por el norte en la Península Ibérica (cuadrante nororiental) y alcanzando hasta los 2.200 m de altitud. La introducción de esta subespecie en cultivos de invernadero en el sur y en el levante ibéricos ha favorecido más ampliamente su dispersión peninsular. Su hallazgo en Sierra Nevada no es sorprendente, aunque sí la altitud (hasta 3.400 m) a la que hemos encontrado algunos ejemplares.

Intentamos sin éxito secuenciar la región COI-COII en el estudio piloto. No obstante, un estudio previo indicó una bajísima diversidad (tres haplotipos) de esta especie en Europa (ESTOUP *et al.* 1996). Ello nos hizo desistir en la búsqueda de diversidad genética.

Apis mellifera Linnaeus, 1758

La abeja de la miel está presente en Sierra Nevada desde tiempos muy remotos tanto de forma natural como introducida. Su manejo ha sido hasta nuestros días muy intenso en las zonas bajas de Sierra Nevada, si bien no hay colmenas registradas en el PNSN. No obstante, los hábitos de desplazamientos diarios en altitud no nos permiten distinguir si los 56 ejemplares capturados son silvestres o pertenecen a colmenas, incluso en el caso de los ejemplares capturados por encima de 3.200 m. Los caracteres morfológicos de todos los ejemplares nos conducen a reconocer únicamente la subespecie *iberiensis* en el PNSN. En concreto, nos estamos refiriendo principalmente a las siguientes características: pubescencia poco abundante, corta y en tono pardo y el aspecto oscuro de la cutícula; terguitos con rasgos característicos como la presencia en el se-

gundo terguito gastral de una banda amarillenta proximal, aunque de tamaño variable que va desde solo dos manchas laterales hasta llegar a cubrir la totalidad del terguito.

Por otra parte, se trata de un taxon endémico ibérico (ORNOSA & ORTIZ-SÁNCHEZ, 2004), si bien todavía no se ha encontrado su identidad filogenética (monofilia) (FRANCK *et al*., 1998; HAN *et al*., 2012). La región mitocondrial COI-COII fue amplificada satisfactoriamente, a pesar de no haber ningún estudio de diversidad genética de esta conocida especie empleando secuencias de esta región (aunque sí con RFLPs, CÁNOVAS *et al*., 2007). Los resultados se muestran más adelante.

Chalicodoma parietina (Geoffroy, 1785)

Abeja totalmente negra (Lámina 1), de tamaño grande (c. 3 cm de largo), que pertenece a una de las familias mejor representadas en la Península Ibérica (Megachilidae). En Iberia se distribuye principalmente en Sierra Nevada (2.100-3.300 m), aunque se citó de zonas más bajas de Almería y Portugal (ORNOSA *et al*., 2007; ORTIZ-SÁNCHEZ *et al*., 2012). La población nevadense de esta especie solitaria de abeja parece estar bien asentada, ya que hemos observado un número considerable de individuos en vuelo entre 2.500 y 3.400 m desde El Veleta hasta el Puntal de los Cuartos (no se observó en El Caballo).

Desgraciadamente no pudimos amplificar la región mitocondrial COI-COII. Ello no nos sorprende debido a que no se han conseguido hasta la fecha secuencias de esta región para esta familia a pesar de que comprende miles de especies. Tampoco conocemos ningún estudio de diversidad genética en una región geográfica (filogeografía) en el que se hayan empleado otras regiones de DNA. Ello nos hizo desistir en la inclusión de esta especie en el estudio genético.

Diversidad genética en la alta montaña nevadense

Finalmente se realizó y analizó un estudio genético extensivo de poblaciones de Sierra Nevada de cinco especies, cuatro de Antirrhineae (*Antirrhinum rupestre*, *Chaenorhinum glareosum*, *Linaria glacialis* y *Linaria nevadensis*) y una especie de abeja (*Apis mellifera*).

Antirrhinum rupestre Boiss. & Reut. (A. hispanicum Chav.)

Se trata de una especie dudosa que se reconocía para la zona antes de iniciar el proyecto (MELENDO *et al*., 2003), pero durante la realización del presente estudio se desestimó su valor taxonómico. Para la Flora vascular de Andalucía oriental, BLANCA *et al*., (2009) incluyeron esta especie como sinónimo de *A. hispanicum*, especie de una distribución mucho más amplia que las inmediaciones de Sierra Nevada. Un criterio taxonómico similar encontramos en la revisión más reciente del género *Antirrhinum* para la Península Ibérica (GÜEMES, 2009), donde también se sinonimizó a *A. hispanicum*. No obstante, algún otro investigador con experiencia en el género indica que esta especie no se ha estudiado bien y por tanto el actual tratamiento taxonómico es incorrecto (Fernández Casas, comunicación personal). Dados los tratamientos taxonómicos incongruentes, hicimos un esfuerzo en evaluar el valor sistemático de la especie basado en la filogenia al incluir muestras del PNSN en una matriz mayor publicada por nuestro equipo (VARGAS *et al*., 2009).

Para el estudio de diversidad genética interpoblacional se analizaron 39 muestras (individuos) de cinco poblaciones para analizar los niveles de diversidad genética (Figura 1). Fue difícil encontrar localidades distantes dentro de los límites del PNSN (Figura 1). Se realizó un amplio estudio piloto de 20 regiones del DNA plastidial. Finalmente se seleccionaron y secuenciaron las dos regiones del plasto (*rpl*32-*trn*L^{UAG}, *trn*S-*trn*G) que dieron variabilidad y señal evolutiva máximas. Para comprobar el valor taxonómico de la especie, realizamos un análisis filogenético con dichas regiones plastidiales donde se obtuvieron tres linajes y se puso de manifiesto la falta de monofilia (resultados no mostrados). Por tanto, según estos resultados la especie perdería valor, ni siquiera como subendemismo de Sierra Nevada. Estos resultados

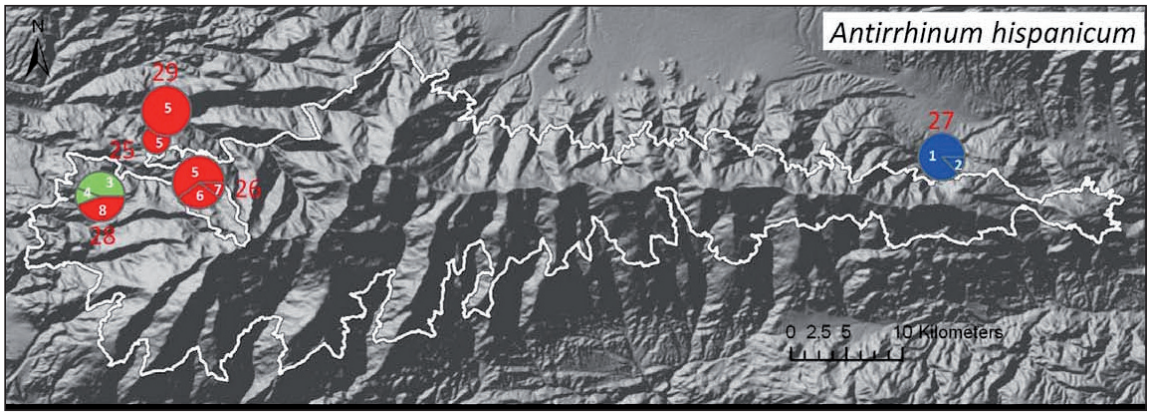


Figura 1. Distribución geográfica de los tres linajes (azul, verde y rojo) de *Antirrhinum rupestre* (considerado finalmente en *A. hispanicum*) y sus respectivos haplotipos. Obsérvese que hay dos poblaciones (no. 25, 29) con un haplotipo, una (no. 27) con dos haplotipos y otras dos con tres haplotipos (nos. 26, 28). Precisamente la única población (Trevenque, no. 28) dentro del PNSN es la que presenta máxima diversidad con tres haplotipos pertenecientes a dos linajes.

Figure 1. Geographical distribution of the three lineages (blue, green and red) and haplotypes of Antirrhinum hispanicum (circunscribed A. hispanicum eventually). Notice that two populations (no. 25, 29) have one haplotype, one (no. 27) has two haplotypes and two more have three haplotypes (nos. 26, 28). The only population (Trevenque, no. 28) within the National park has the highest diversity including three haplotypes of two lineages.

coinciden con los resultados taxonómicos comentados más arriba.

Aun así, realizamos un análisis de diversidad genética con las secuencias obtenidas de nuestro muestreo en el PNSN (y proximidades). Se han encontrado tres linajes diferentes de dos, dos y cuatro haplotipos (véanse colores en la Figura 1). La distribución de la diversidad genética tanto en número de haplotipos (8) como de linajes (un linaje del este y dos linajes del oeste) tiene un fuerte componente geográfico, tal y como ya se ha descrito para el género *Antirrhinum* (VARGAS *et al*.,, 2009). En cualquier caso, la población más diversa por número de haplotipos (3) y linajes (2) se ha localizado en el área del Trevenque (Figura 1). Resulta interesante señalar que precisamente esta población es la única localidad situada dentro del PNSN.

Chaenorhinum glareosum (Boiss.) Willk.

En este caso se trata de una especie muy bien definida ya que no se han encontrado incongruencias taxonómicas de importancia. Además, su aislamiento geográfico (piso de vegetación crioromediterráneo de Sierra Nevada) da garantías de ser un buen bioindicador de diversidad genética vegetal en el PNSN para las cotas entre 2700 y 3400 m. Finalmente se analizaron 94 muestras (individuos) de 11 poblaciones para analizar los niveles de diversidad genética y se seleccionaron y secuenciaron las dos regiones del plasto (*rpl*32-*trnL*^{UAG} y *trnQ*-*rps*16) que dieron gran variabilidad y señal filogeográfica.

Un total de 11 haplotipos fueron detectados en las poblaciones de *Chaenorhinum glareosum*. La relación entre los haplotipos es compleja y no se obtuvo una estructura clara entre los mismos (resultados no mostrados). No obstante, los cinco linajes menores detectados que se formaron a partir un haplotipo central (azul) nos permiten interpretar la diversidad genética de la especie tanto por la distribución geográfica de los haplotipos como por la distribución geográfica de esos cinco linajes. Por otra parte, esa elevada diversidad genética tiene una amplia distribución geográfica. Por ejemplo, dos haplotipos separados por solo dos mutaciones se han localizado en poblaciones extremas: Tozal del Cartujo al oeste (haplotipos negro y morado) y Puntal de Trévelez al este (haplotipos negro y azul marino) (Figura 2). Y no solo esto. Las poblaciones

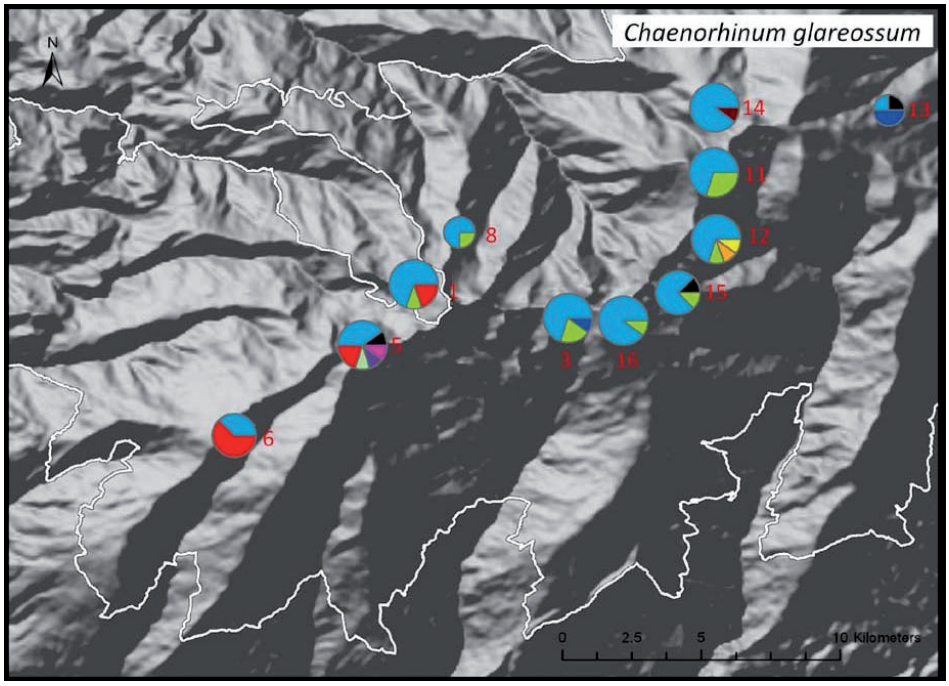


Figura 2. Distribución geográfica de los haplotipos de *Chaenorhinum glareosum*. Obsérvense varios colores (con sus tonalidades) que corresponden a varios linajes menores como consecuencia de la estructura de relación evolutiva entre haplotipos. La máxima diversidad haplotípica se ha encontrado en la población de El Tozal del Cartujo (no.5). La diversidad máxima de linajes (el haplotipo central más dos linajes) se localizó no solo en esta población (no.5), sino también en el Veleta (no.1), Mulhacén (no.3), Culo Perro (no.15) y Puntal de Vacares (no.12).

Figure 2. Geographical distribution of the haplotypes of Chaenorhinum glareosum. Notice different colours indicating sublineages as a result of evolutionary relationships of the haplotypes. The highest haplotype diversity was found in El Tozal del Cartujo population (no.5). The highest haplotype-lineage diversity (the central haplotype plus two lineages) was found not only in population no. 5 but also in el Veleta (no. 1), Mulhacén (no.3), Culo Perro (no.15) and Puntal de Vacares (no.12).

muestreadas albergan un número de haplotipos elevado (entre dos y seis), lo que indica una riqueza genética por población muy elevada.

Precisamente esta elevada diversidad genética nos ha permitido señalar la población más rica (Tozal del Cartujo) con seis haplotipos y tres linajes, seguida de otra de cuatro haplotipos y dos linajes (Puntal de Vacares) y por último tres poblaciones con tres haplotipos y dos linajes (Veleta, Mulhacén y Culo Perro) (Figura 2). Por el contrario, otra población del Mulhacén (no.16) y Cerro de San Juan y Pico del Cuervo mostraron solo dos haplotipos muy próximos (una sola mutación). Además, esos dos haplotipos son los mismos y bien distribuidos en todas (azul claro) o casi todas (verde) las poblaciones de Sierra Nevada.

Todos estos resultados indican que la especie tiene una gran diversidad genética, que hay poca estructuración geográfica de la diversidad y que ciertas áreas son más ricas que otras, pero a pequeña escala (no observamos un patrón geográfico latitudinal o longitudinal claro en Sierra Nevada, pongamos por caso). Es decir, ciertas áreas albergan poblaciones muy pobres en diversidad genética, mientras que otras albergan una importante diversidad genética tanto en haplotipos como en linajes. Tanto es así que solo dos poblaciones (Tozal del Cartujo y Puntal de Vacares) albergan nueve de los once haplotipos detectados, lo que supone más de un 80% de la diversidad genética de la especie. Es decir, nuestros resultados indican que si conservaramos la diversidad genética de tan solo estas dos poblaciones conservaríamos la mayor parte de la diversidad genética estimada.

Linaria glacialis Boiss.

Al igual que la especie anterior, se trata de una planta bien definida taxonómicamente y sin incongruencias taxonómicas desde su descripción original. También coincide con la especie anterior en un gran aislamiento geográfico (piso de vegetación crioromediterráneo de Sierra Nevada). En este caso su límite altitudinal inferior (cotas por encima de 3000 m) es incluso más elevado (BLANCA *et al.*, 1988), por lo que estamos ante uno de los endemismos más interesantes para la reconstrucción de la historia evolutiva de Sierra Nevada y para el análisis de cambio climático. Por si esto fuera poco, esta especie ha sido catalogada como "vulnerable" en el catálogo de flora amenazada de Andalucía (BOJA nº 60 de 27/03/2012).

Finalmente se analizaron 106 muestras (individuos) de 16 localidades para analizar los niveles de diversidad genética. A falta de estudios de flujo génico, algunas de estas poblaciones podrían considerarse subpoblaciones dentro de un mismo macizo montañoso dada su proximidad geográfica, por lo que finalmente consideramos 10 poblaciones para los análisis (Figura 3). Se seleccionaron y secuenciaron las dos regiones del plasto (*rpl*32-*trnL*^{UAG} y *rps16*-*trnK*) que daban mayor variabilidad y mayor señal evolutiva. Se detectó un total de 19 haplotipos en las poblaciones de *Linaria glacialis*. La relación evolutiva entre los haplotipos está muy bien estructurada, lo que indica procesos pretéritos de aislamiento dentro de Sierra Nevada. En concreto, se describen claramente dos linajes: haplotipos 1-10 (en negro) y linaje 11-19 (en blanco) (Figura 3).

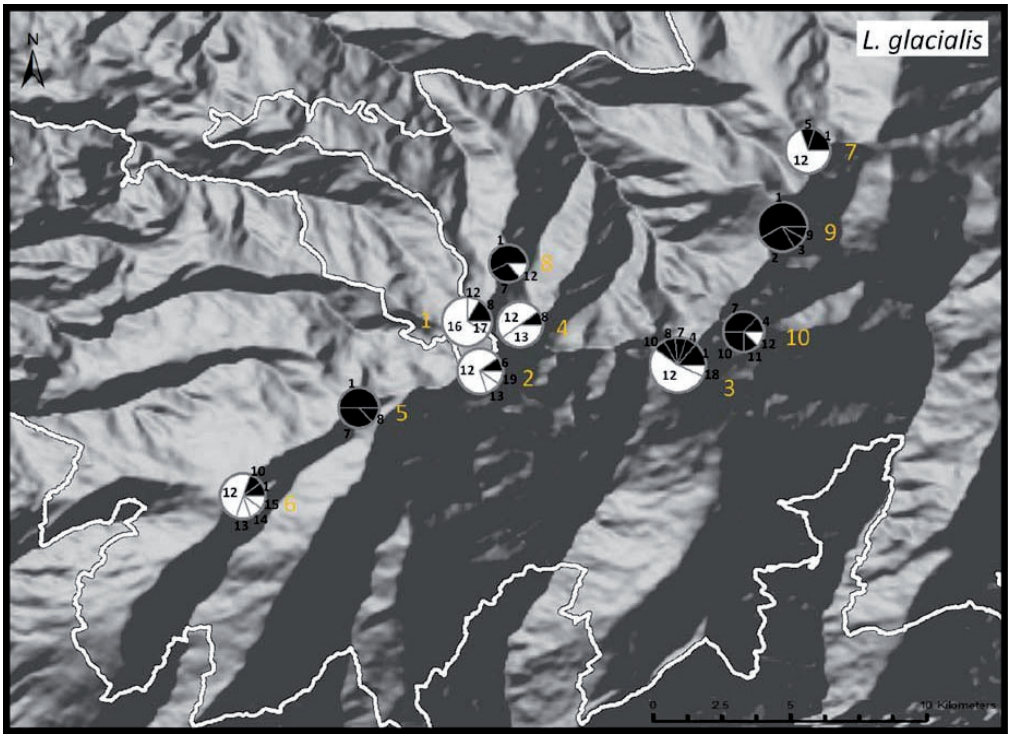


Figura 3. Distribución geográfica de los 19 haplotipos y linajes (en blanco y negro) de *Linaria glacialis*. Obsérvense las máximas diversidades haplotípicas de siete y seis haplotipos encontradas en la población de El Caballo (no.6) y Mulhacén (no.3). Por el contrario, otras cuatro poblaciones solo mostraron tres haplotipos.

Figure 3. Geographical distribution of the 19 haplotypes and two lineages (black and white) of Linaria glacialis. Notice that the highest haplotype diversity was found in *El Caballo* (no.6) and *Mulhacén* (no.3) based on seven and six haplotypes, respectively. In contrast, four populations harboured only three haplotypes.

El número de haplotipos por población fluctúa entre tres y siete. La población con el número más elevado de haplotipos es la del Mulhacén (no. 3) con siete haplotipos, seguida de El Caballo (no. 6) con seis haplotipos y el Pico del Globo (no. 10) con cinco haplotipos. El bajo número de haplotipos de ciertas poblaciones no coincide necesariamente en todos los casos con una baja diversidad de linajes, dado que algunas poblaciones tienen un bajo número de haplotipos (tres), pero de los dos linajes: Cerro de los Cuartos (no. 4), Puntal de los Cuartos (no. 7) y Cerro de San Juan (no. 8). Cuando tenemos en cuenta los resultados de diversidad haplotípica (número de haplotipos) y de la diversidad de linajes (número de linajes) en su conjunto, los dos análisis señalan al macizo del Mulhacén como el área de mayor riqueza. Es más, la diversidad de linajes está presente en el macizo del Mulhacén en similares porcentajes (linaje blanco c. 60% y el negro c. 40%).

Todos estos resultados sugieren que ciertas áreas, y no amplias regiones de Sierra Nevada, albergan gran riqueza genética. Además, nuestros resultados indican importantes diferencias de diversidad genética entre áreas de manera que unas pocas áreas albergan un porcentaje muy considerable de la diversidad total. Es decir, si conservamos la diversidad genética de tan solo las poblaciones del Mulhacén y del Puntal de los Cuartos conservaríamos 10 haplotipos de los dos linajes, lo que supone más del 50 % de la diversidad total estimada de la especie.

En cualquier caso, pese a su buena salud genética, se prevén unas consecuencias catastróficas para esta especie en el próximo siglo debido al cambio climático (BLANCO-PASTOR *et al.*, 2013).

***Linaria nevadensis* (Boiss.) Boiss. & Reut.**

A diferencia de las dos anteriores, esta especie sí que ha sufrido distintos tratamientos taxonómicos como especie (*Linaria nevadensis*) o subespecie (*Linaria aeruginea* subsp. *nevadensis*). El último tratamiento taxonómico (SÁEZ & BERNAL, 2009) adopta este último criterio. Por otro lado, esta especie representa unas cotas altitudinales diferentes a las de las especies anteriores (piso de vegetación oromediterráneo de Sierra Nevada). En concreto, este taxon se desarrolla principalmente en la franja entre 2.300 y 3.300 m.

Se analizaron 56 muestras (individuos) de 14 poblaciones para analizar los niveles de diversidad genética (Figura 4). Finalmente se seleccionaron y secuenciaron las dos regiones del plasto (*rpl*32-*trnL*^{UAG} y *trnQ-rps*16) que dieron variabilidad y señal evolutiva máximas. Un total de 21 haplotipos fueron detectados en las poblaciones de *Linaria nevadensis*. La relación evolutiva entre los haplotipos está muy bien estructurada, y es muy similar a la de la especie anteriormente descrita (*Linaria glacialis*). Igualmente interpretamos procesos pretéritos de aislamiento dentro en Sierra Nevada. En concreto, se describen claramente dos linajes, si bien uno de ellos (números con fondo negro) solo está formado por los haplotipos 1-5 y el otro (en blanco) por los haplotipos 6-21 (Figura 4).

Asimismo, esta elevada diversidad genética tiene una amplia distribución geográfica (Figura 4). Se trata del taxon que tiene una diversidad aun más repartida geográficamente. Un haplotipo se encuentra en toda Sierra Nevada (el haplotipo 6 está en todas las poblaciones). La diversidad haplotípica intrapoblacional varía entre uno (Hoya de la Mora, no. 20 y San Juan, no. 24) y cinco haplotipos. Las poblaciones con máxima diversidad haplotípica (cinco haplotipos) son El Veleta (no. 1) y el Pico del Cuervo (no. 11), seguidas del Puntal de Vacares (no. 12) con cuatro haplotipos. Otras poblaciones de las que se han tomado 5-6 muestras no han dado proporcionalmente una alta variabilidad genética: Tozal del Cartujo (dos haplotipos), San Juan (un haplotipo), Acucaderos (tres haplotipos) y Puntal de los Cuartos (tres haplotipos).

También se puede deducir una buena distribución geográfica de linajes al observar que seis localidades tienen haplotipos de los dos linajes (negro y blanco). Además, el bajo número de haplotipos de ciertas poblaciones no coincide necesariamente en todos los casos con una baja

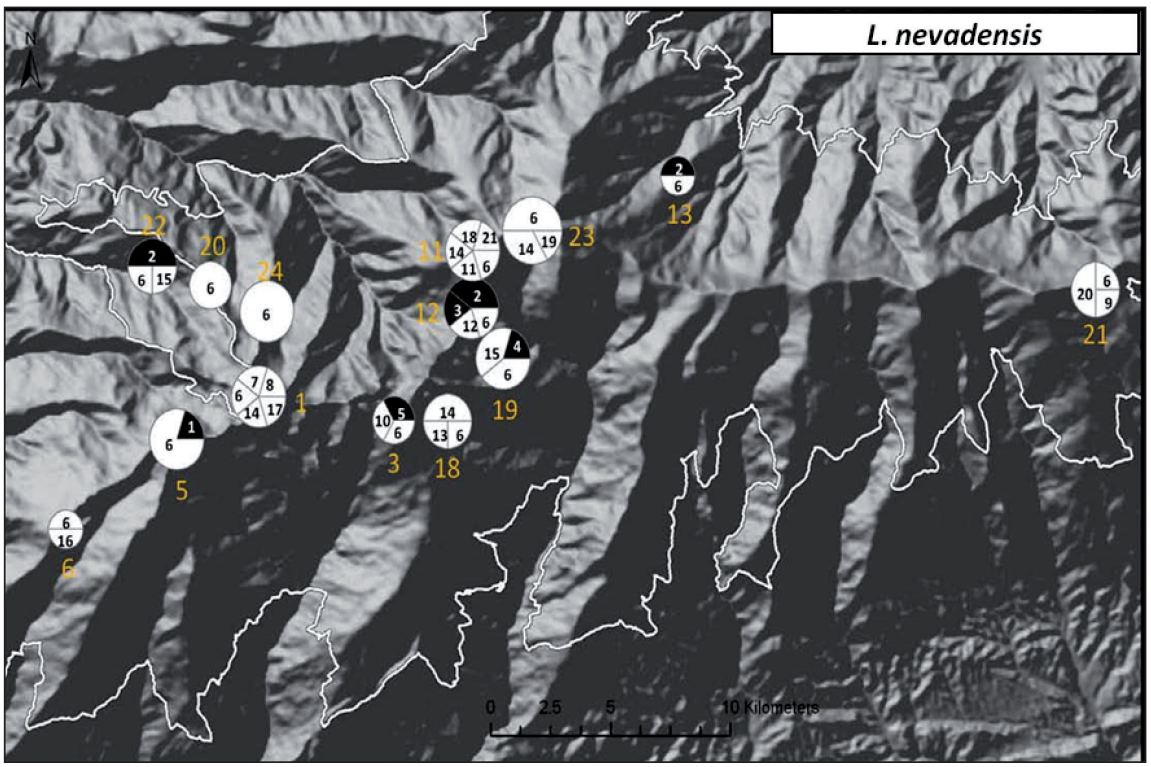


Figura 4. Distribución geográfica de los 21 haplotipos y linajes (en blanco y negro) de *Linaria nevadensis* (considerada *L. aeruginea* subsp. *nevadensis* por algunos autores). Obsérvese que la diversidad genética está bastante bien distribuida en todo el Parque Nacional.
Figure 4. Geographical distribution of the 21 haplotypes and two lineages (black and white) of Linaria nevadensis (considered as L. aeruginea subsp. nevadensis by some authors). Notice that the genetic diversity of this species is well distributed across the National Park.

diversidad de linajes. Por ejemplo, las poblaciones del Tozal del Cartujo (no. 5) y Puntal de Trévelez (no. 13) solo presentan dos haplotipos, pero de los dos linajes. Cuando tenemos en cuenta los resultados de diversidad haplotípica (número de haplotipos) y de la diversidad de linajes (número de linajes) en su conjunto, es difícil señalar ciertas poblaciones que muestren altos índices de diversidad para ambos (Figura 4). No obstante, la población que muestra haplotipos de los dos linajes y un alto número de haplotipos es la del Puntal de Vacares (no. 12). Esta especie también se ajusta a una tendencia generalizada a que ciertas poblaciones son especialmente ricas en diversidad genética, mientras que otras no albergan diversidad genética (no. 20, no. 24). Por ello, si conservamos la diversidad genética de ciertas poblaciones podríamos preservar más del 50% de la diversidad total de la especie.

Apis mellifera (Linnaeus, 1978) subsp. *iberiensis* (Engel, 1999)

Se trata de un taxon endémico de la Península Ibérica. Según algunos autores es un eslabón entre las abejas europeas pertenecientes a la subsp. *mellifera* y subespecies norteafricanas (ORNOSA & ORTIZ, 2004; aunque véase FRANCK *et al*., 1998). Estudios previos han detectado tres linajes en las poblaciones de toda la Península Ibérica: linaje A, que es africano; linaje C, de Europa oriental; y linaje M, de Europa occidental. Un análisis reciente de la distribución de haplotipos mitocondriales de 27 poblaciones ibéricas arroja un resultado de un 61,67% del linaje africano, 1,67% del linaje de Europa oriental y 36,67% del linaje de Europa occidental (CÁNOVAS *et al*., 2007; DE LA RUA *et al*., 2009). En concreto, se ha descrito que en la provincia de Granada el linaje predo-

minante sería el africano (linaje A, sublinaje AI), que tendría una presencia de un 80%. Dado que el PNSN está tan próximo a África, pero las cotas de estudio son elevadas y coinciden en condiciones climáticas con regiones norteñas de la Península Ibérica, la cuestión es si las poblaciones del PNSN están formadas por individuos del linaje europeo, del africano o de ambos.

Se analizaron 30 muestras (individuos) de seis poblaciones distantes para analizar los niveles de diversidad genética (Figura 5). El muestreo de poblaciones fue menor que el inicialmente previsto. En cualquier caso se amplió el muestreo con dos poblaciones tomadas fuera del PNSN donde hay predominancia del linaje europeo de *Apis mellifera* (Guadalajara) y del linaje africano (Almería) para obtener información sobre el origen de los linajes haplotípicos. El espaciador mitocondrial COI-COII proporcionó un tamaño de 563 bp.

Un total de 21 haplotipos fueron detectados en las poblaciones de *Apis mellifera* subsp. *iberiensis*. La relación evolutiva entre los haplotipos tiene una estructura principalmente lineal, si bien se distinguen tres grupos de haplotipos (azul, verde y rojo) (Figura 5). El grupo azul (13 haplotipos) y el grupo verde (tres haplotipos) están separados del rojo por más de 10 haplotipos extintos o no encontrados (*missing*). Por tanto, el grupo rojo es el más separado con respecto a los otros dos. Al estar separados ambos grupos por tantas mutaciones nucleotídicas deducimos la presencia de los dos linajes (africano y europeo) en las poblaciones del PNSN. Estos resultados coinciden con los dos linajes, africano (A) y europeo occidental (M), descritos para esta zona de la Península Ibérica (véase más arriba; DE LA RUA *et al.*, 2009). De hecho, haplotipos del grupo azul se han encontrado en las muestras de Almería empleadas como grupo externo y haplotipos del grupo rojo

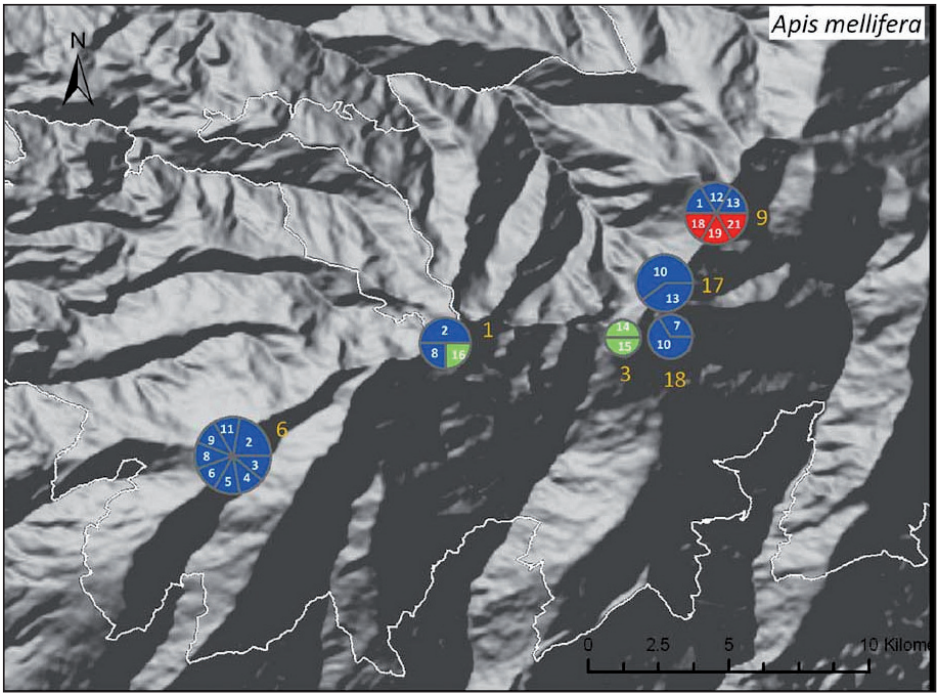


Figura 5. Distribución geográfica de los 19 haplotipos y linajes (en azul, rojo y verde) de *Apis mellifera iberiensis*. Obsérvese la estructuración geográfica de la diversidad genética basada en seis poblaciones: El Caballo (no. 6), Veleta (no. 1), Mulhacén (no. 3), Tajos del Goterón (no. 17), Laguna Hondera (no. 18) y Pico del Cuervo-Puntal de Vacares (no. 9).

Figure 5. Geographical distribution of the 19 haplotypes and three lineages (blue, red and green) of Apis mellifera iberiensis. Notice the geographical structure of the genetic diversity for six populations: El Caballo (no. 6), Veleta (no. 1), Mulhacén (no. 3), Tajos del Goterón (no. 17), Laguna Hondera (no. 18) and Pico del Cuervo-Puntal de Vacares (no. 9).

en las muestras de Guadalajara. Además, el patrón encontrado de sublinajes (azul y verde) coincide con los dos sublinajes (AI y AIII) detectados con anterioridad para Granada (DE LA RUA *et al.*, 2009). Este encuentro de tres linajes ha enriquecido de manera muy significativa la diversidad genética de *Apis mellifera* en Sierra Nevada. Además, el linaje azul es especialmente rico en esta sierra (13 haplotipos).

La distribución de la diversidad genética de haplotipos y linajes es también muy rica para esta especie. Las dos poblaciones de los extremos este (Pico del Cuervo/Puntal de Vacares, no. 9) y oeste (El Caballo, no. 6) son las más ricas en número de haplotipos con seis y ocho respectivamente (Figura 5). Las demás poblaciones contienen 2-3 haplotipos, si bien se dispuso de un menor número de individuos de alguna población (Figura 5). También encontramos cierta estructuración geográfica, con el linaje rojo solo en el noreste, el verde solo en el centro y el azul a lo largo de toda la Sierra. Por tanto la población con mayor número de haplotipos y linajes se localizó en el Pico del Cuervo/Puntal de Vacares, que además no está lejos de otra localidad con haplotipos del linaje verde (Mulhacén, no. 3). Por tanto se ha localizado una región (Mulhacén-Puntal de Vacares) con más de la mitad (14) de los haplotipos encontrados y de los tres linajes.

DISCUSIÓN

Utilidad de las herramientas genéticas en conservación

Las principales aproximaciones que se han empleado hasta la fecha para identificar áreas de máximo interés en Sierra Nevada se han basado en la riqueza de hábitats y de número de especies. El presente estudio representa un nuevo enfoque en el que se localizan áreas que albergan una elevada diversidad genética. Se trata, por tanto, de un enfoque complementario a los anteriores y de especial interés en ambientes de alta montaña, donde los hábitats son similares, las comunidades de roquedo y pedreras son dominantes y muy sensibles ante el cambio climático.

En definitiva, nuestro enfoque propone las siguientes consideraciones a ser estudiadas para una gestión y conservación más precisas de la flora y fauna de montaña:

(1) Las técnicas de *barcoding* sirven para proporcionar un elevado número de marcadores moleculares y unas regiones de DNA de gran confianza evolutiva, por lo que confieren una herramienta de conservación muy valiosa para detectar la "criptodiversidad" (o biodiversidad escondida) dentro de una misma especie.

(2) Las tres plantas endémicas de Sierra Nevada estudiadas (*Chaenorhinum glareosum*, *Linaria glacialis* y *Linaria nevadensis*) tienen unos niveles de diversidad genética muy alta (en comparación con otras especies de los mismos géneros fuera de Sierra Nevada). Ello nos permite ser optimistas en cuanto al patrimonio genético de las especies.

(3) Las dos especies de más amplia distribución (*Apis mellifera iberiensis* y *Antirrhinum hispanicum*) también presentan elevados niveles de diversidad, si bien esta puede ser debida a antiguos contactos entre diferentes linajes ibéricos.

(4) La combinación de plantas y sus polinizadores no solo ha permitido demarcar áreas de interés, sino también valorar la importancia ecológica de organismos interdependientes e imprescindibles para el funcionamiento de los ecosistemas.

(5) El análisis conjunto de varias especies permite encontrar áreas más consistentes que por separado. Sería deseable seguir estudiando la diversidad genética de otras especies de distintos tipos de organismos para poder señalar las áreas de máximo interés biológico en Sierra Nevada.

Áreas de mayor diversidad

Diferentes poblaciones han mostrado diferentes niveles de diversidad genética para cada especie. Este resultado implica de por sí que no hay áreas que alberguen la máxima diversidad genética para todas las especies. Tampoco se han obser-

vado máximos niveles de diversidad genética en regiones amplias de Sierra Nevada (suma de áreas del este, oeste, norte o sur del PNSN) para ninguna de las especies. Sin embargo, al combinar los resultados genéticos de las especies de plantas (*Chaenorhinum glareosum*, *Linaria glacialis* y *Linaria nevadensis*) y de abeja (*Apis mellifera*) sobre las que se hicieron los estudios extensivos en toda Sierra Nevada podemos demarcar localidades que aportan una diversidad genética alta, media y baja (Figura 6). El criterio para proponer estas áreas se basa en el mínimo número de poblaciones que alberguen una diversidad igual o superior al 50 % de cada especie en Sierra Nevada. Para ello se ha considerado tanto la diversidad de haplotipos como de linajes haplotípicos. Una vez localizadas estas áreas para cada especie, se han combinado los mínimos números de poblaciones necesarios para albergar una diversidad igual o superior al 50% para dos o más especies.

En concreto, las siguientes poblaciones suponen una diversidad genética igual o superior al 50% del total de cada especie: para *Chaenorhinum glareosum* la suma de las diversidades genéticas de las poblaciones del Tozal del Cartujo y Puntal de Vacares; para *Linaria glacialis* la suma de las del Mulhacén y El Caballo; para *Linaria nevadensis* la suma de las de Pico del Cuervo, Puntal de Vacares y Acucaderos; y para *Apis mellifera iberiensis* la suma de las de El Caballo y Pico del Cuervo.

En la Figura 6 se muestran las áreas con distintos niveles de diversidad genética: máxima cuando hay dos áreas de diversidad máxima para dos especies (en rojo), media cuando hay una sola área de diversidad máxima de una especie (en naranja) y baja cuando las poblaciones no albergan una diversidad genética elevada de ninguna especie (en verde). Esta síntesis de la diversidad genética a lo largo de Sierra Nevada indica que tres áreas (poblaciones) deben ser señaladas como las más ricas: El Caballo, Puntal de Vacares y Pico del Cuervo. Resulta de interés señalar que las tres áreas no coinciden necesariamente con áreas de máxima diversidad estimada por el número de especies de angiospermas, tales como El Veleta o la Hoya de la Mora.

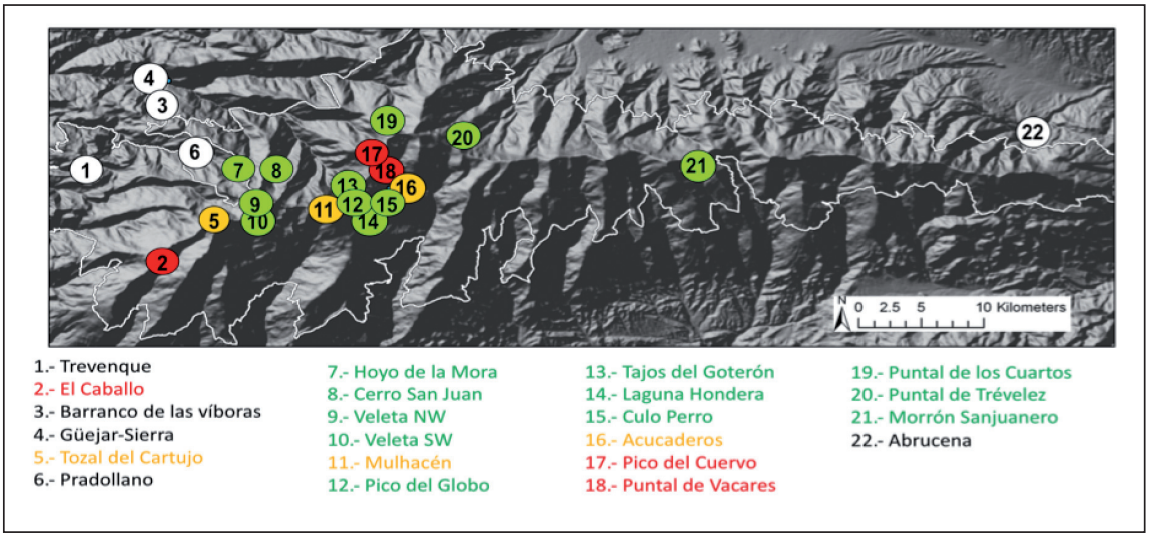


Figura 6. Áreas de diversidad genética obtenidas de la combinación de resultados de las tres especies de plantas y una de abeja empleadas. Se marca con círculos rojos las poblaciones que muestran diversidad máxima para dos especies, en naranja las que muestran diversidad máxima para solo una especie y en verde las que no albergan una diversidad genética elevada. Círculos con fondo blanco indican las poblaciones no utilizadas para este análisis.

Figure 6. Areas of genetic diversity based on the combination of results from three plant and one bee species. Red circles indicate populations with the highest diversity based on two species, orange circles for that based on one species and green circles with no species with the highest diversity. White circles indicate populations eventually not used for the analysis.

En definitiva, nuestros resultados no apuntan a regiones (conjuntos de áreas) dentro del PNSN con especial interés, sino a áreas más locales donde se encuentra gran diversidad genética. Ello nos sugiere que una política de microrreservas sería la estrategia más interesante a la hora de priorizar medios y esfuerzos. En concreto, una política de las microrreservas dentro de Sierra Nevada sobre la base de la diversidad de hábitats, número de especies y diversidad genética permitiría preservar el patrimonio de este Parque Nacional de una manera más eficiente y a un menor coste.

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REFERENCIAS BIBLIOGRÁFICAS

BLANCA, J., CABEZUDO, B., CUETO, M., FERNÁNDEZ-LÓPEZ, C. & MORALES-TORRES, C. 2009. Flora vascular de Andalucía Oriental, 4 vols. Consejería de Medio Ambiente, Junta de Andalucía, Sevilla.

BLANCA, J., CUETO, M., MARTÍNEZ-LIROLA & M.J., MOLERO-MESA, J. 1998. Threatened vascular flora of Sierra Nevada (Southern Spain). *Biological Conservation* 85: 269-285.

BLANCO-PASTOR, J.L., VARGAS, P. & PFEIL, B.E. 2012. Coalescent simulations reveal hybridization and incomplete lineage sorting in Mediterranean *Linaria*. *PLoS ONE* 7: e39089.

BLANCO-PASTOR, J.L., FERNÁNDEZ-MAZUECOS, M. & VARGAS, P. 2013. Past and future demographic dynamics of alpine species: limited genetic consequences despite dramatic range contraction in a plant from the Spanish Sierra Nevada. *Molecular Ecology*. 22: 000-000.

CÁNOVAS, F., DE LA RÚA, P., SERRANO, J., & GALIÁN, J. 2007. Geographical patterns of mitochondrial DNA variation in *Apis mellifera iberiensis* (Hymenoptera: Apidae). *Journal of Zoological Systematics and Evolutionary Research* 46: 24–30.

CORNUET, J.M., GARNERY,L. & SOLIGNAC, M. 1991. Putative origin and function of the intergenic region between COI and COII of *Apis mellifera* L. mitochondrial DNA. *Genetics* 128: 393-403.

DE LA RÚA, P., MUÑOZ, I., PIÑERO, A., HERNÁNDEZ-GARCÍA R., CÁNOVAS, F., GALIÁN, J. & SERRANO, J. 2009. Historia evolutiva de la abeja ibérica. En: Adaptación y evolución. 150 años después del Origen de las Especies. Pp: 162-169. SESBE. Valencia.

EBMER, A.W. 2003. Hymenopterologische Notizen aus Österreich 16 (Insecta: Hymenoptera: Apoidea). *Linzer biologische Beiträge* 35: 313-403.

ESTOUP, A., SOLIGNAC, M., CORNUET, J.-M., GOUDET, J. & SCHOLL, A. 1996. Genetic differentiation of continental and island populations of *Bombus terrestris* (Hymenoptera: Apidae) in Europe. *Molecular Ecology* 5: 19-31.

FERNÁNDEZ-MAZUECOS, M. & VARGAS, P. 2011. Historical Isolation versus Recent Long-Distance Connections between Europe and Africa in Bifid Toadflaxes (*Linaria* sect. Versicolores). *PLOS ONE* 6(7): e22234.

FRANCK, P., GARNEY, L., SOLIGNAC, M. & CORNUET, J.-M. 1998. The Origin of West European Subspecies of Honeybees (*Apis mellifera*): New Insights from Microsatellite and Mitochondrial Data. *Evolution* 52: 1119-1134.

GIBSON, G. & DWORKIN, I. 2004. Uncovering cryptic genetic variation. *Nature Reviews Genetics* 5: 681-690.

GÓMEZ, J.M. & ZAMORA, R. 1999. Generalization vs. specialization in the pollination system of *Hormatophylla spinosa* (Cruciferae). *Ecology* 80: 796-805.

GÜEMES, J. 2009. *Antirrhinum* L. En: Castroviejo, S., Benedí, C., Rico, E., Güemes, J. y Perret, P. (eds.). Flora ibérica, plantas vasculares de la Península Ibérica e Islas Baleares. Vol. 13. Pp. 134-166. Real Jardín Botánico, CSIC. Madrid.

HAN, F., WALLBERG, A. & WEBSTER, M.T. 2012. From where did the Western honeybee (*Apis mellifera*) originate? *Ecology and Evolution* 2: 1949-1957.

MELENDO, M., GIMÉNEZ, E., CANO, E., GÓMEZ-MERCADO, F. & VALLE, F. 2003. The endemic flora in the south of the Iberian Peninsula: taxonomic composition, biological spectrum, pollination, reproductive mode and dispersal. *Flora - Morphology, Distribution, Functional Ecology of Plants* 198: 260-276.

ORNOSA, C. & ORTIZ-SÁNCHEZ, F.J. 2004. Hymenoptera, Apoidea I. En: Fauna Ibérica, vol. 23. Ramos, M.A. et al., (eds.). Museo Nacional de Ciencias Naturales. CSIC. Madrid. 556 pp.

ORNOSA, C., ORTIZ-SÁNCHEZ, F.J. & TORRES, F. 2007. Catálogo de los Megachilidae del Mediterráneo occidental (Hymenoptera, Apoidea). II. Lithurgini y Megachilini. *Graellsia* 63: 111-134.

ORNOSA, C., TORRES, F. & ORTIZ-SÁNCHEZ, F.J. 2009. Claves y datos nuevos de las especies ibéricas del género *Stelis* Panzer, 1806 (Hymenoptera, Apoidea, Megachilidae, Anthidiini). *Graellsia* 65: 111-132.

ORTIZ-SÁNCHEZ, F.J., ORNOSA, C. & TORRES, F. (en prensa). Los apoideos (Hymenoptera, Apoidea) de Sierra Nevada. En: Tinaut, A., Tierno, J.M. y Ruano, F. (eds.). Insectos de Sierra Nevada: Biodiversidad y Conservación. Consejería de Medio Ambiente, Junta de Andalucía. Sevilla.

ORTIZ-SÁNCHEZ, F.J., ORNOSA, C. & TORRES, F. 2012. Especies ibéricas de los géneros *Lithurgus* Berthold, 1827, *Creightonella* Cockerell, 1908 y *Chalicodoma* Lepeletier, 1841 (Hymenoptera, Megachilidae): claves de identificación y nuevos datos de distribución. *Graellsia* 68: 181-206.

RORTAIS, A., ARNOLD, G., ALBURAKI, M., LEGOUT, H. & GARNERY, L. 2011. Review of the DraI COI-COII test for the conservation of the black honeybee (*Apis mellifera mellifera*). *Conservation Genetics Resources* 3: 383-391.

SÁEZ, L. & BERNAL, M. 2009. *Linaria* Mill. En: Benedí, C., Rico, E., Güemes, J. y Perret, P. (eds.). Flora ibérica, plantas vasculares de la Península Ibérica e Islas Baleares. Vol. 13. Pp: 232-324. CSIC, Madrid.

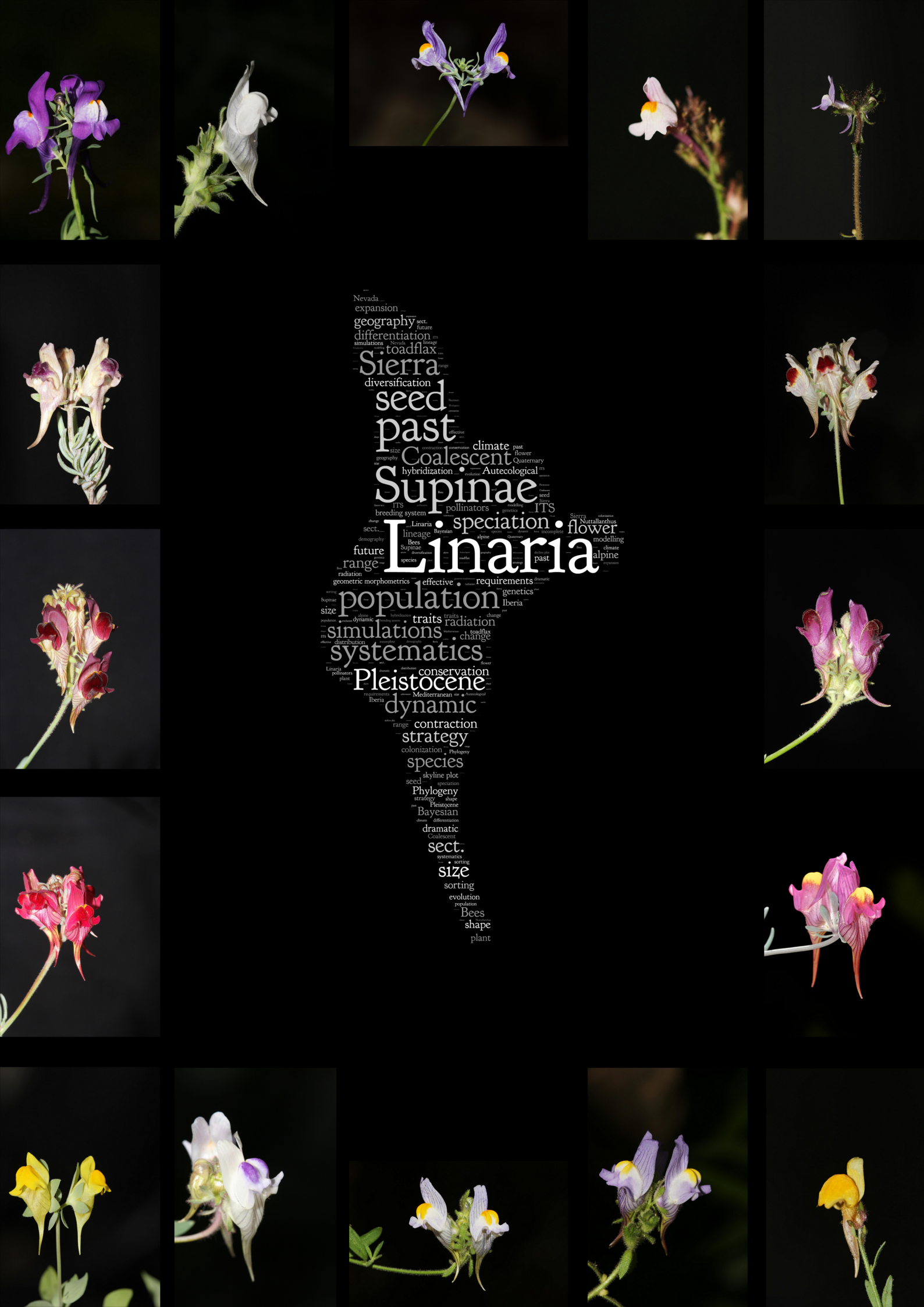
VARGAS, P., CARRIÓ, E., GUZMÁN, B., AMAT, E. & GÜEMES, J. 2009. A geographical pattern of *Antirrhinum* (Scrophulariaceae) speciation since the Pliocene based on plastid and nuclear DNA polymorphism. *Journal of Biogeography* 36: 1297–1312.

VARGAS, P., ORNOSA, C., ORTIZ-SÁNCHEZ, J. & ARROYO, J. 2010. Is the occluded corolla of *Antirrhinum* bee-specialized? *Journal of Natural History* 44: 1427-1443.

WARNCKE, K. 1991. Die Bienengattung *Osmia* Panzer, 1806, ihre Systematik in der Westpaläarktis und ihre Verbreitung in der Türkei. 10. Die Untergattung *Alcidamea* Cress. Linzer biologische Beiträge, 23(2): 701-751.

WARNCKE, K. 1992a. Die westmediterranen Arten der Bienen *Osmia* subg. *Hoplitis* Klug 1807. *Linzer Biologische Beiträge* 24: 103-121.

ZANDEN, G. van der. 1991. Systematik und Verbreitung der paläarktischen Arten der Untergattung *Caerulosmia* van der Zanden 1989 (Hymenoptera, Apoidea, Megachilidae). *Linzer biologische Beiträge* 23: 37-78.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